

Studies on the Metabolism of Catecholamines  
in the Central Nervous System and  
the Action of Drugs thereon.

METHODS FOR THE ESTIMATION OF  
CATECHOLAMINES AND THEIR METABOLITES  
IN THE SAME SAMPLE OF BRAIN  
TISSUE: APPLICATION TO A STUDY  
ON THE EFFECTS OF CHLORPROMAZINE  
ON THE METABOLISM OF CATECHOLAMINES  
IN DOG BRAIN.

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The thesis is divided into two sections.

Section I describes the development of a technique for separating and estimating the catecholamines and their metabolites in a single sample of brain tissue. This section is divided into two sub-sections. In sub-section 1 a method is described in which the amines are separated, prior to estimation, by paper chromatography using phenol : hydrochloric acid. This method proved to be unsuitable for several reasons when applied to brain extracts. A much more satisfactory procedure was developed in which the amines are separated and estimated in the form of their acetylated derivatives. This latter technique and its application to the analysis of extracts from rat brain are described in sub-section 2.

Section II is concerned with an investigation into the effects of chlorpromazine on the metabolism of catecholamines in discrete areas of dog brain, using the analytical techniques described in Section I, Sub-section 2.

## SECTION I

### Methods for the Estimation of Catecholamines and their Metabolites in Brain Tissue

## INTRODUCTION

The first estimations of brain catecholamines were carried out with biological methods and were of necessity limited to the estimation of adrenaline and noradrenaline. Using these methods, the distribution of adrenaline and noradrenaline in brain and the effects of drugs on the amine levels were studied (Vogt, 1954). It was impossible to tell from these experiments if a change in amine concentration following administration of a drug resulted from an effect of the drug on storage, synthesis or metabolism of the amine. Localisation of the sites of drug action only became feasible after the introduction of fluorimetric methods whereby not only adrenaline and noradrenaline but also their precursors and metabolites, could be assayed.

Development of methods for estimating the amines and their metabolites stimulated a search for methods of separating the amines previous to their measurement. Separation techniques have tended to lag behind estimation techniques with the result that the number of amines and their metabolites which can be estimated in one sample has been limited. Deficiencies in separation have to some extent been made good by development of assay methods which are sufficiently specific to allow one compound to be estimated in the presence of another

and sufficiently sensitive to allow division of the sample into as many aliquots as there are compounds to be measured. Thus most of the reports to date on brain amines and their metabolites have been restricted to the estimation of only two or three of these compounds from one brain sample (Baird and Lewis, 1964; Juorio and Vogt, 1965; Bertler and Rosengren, 1959) or have covered a wider range of compounds by pooling brains from several animals and taking aliquots for the estimation of individual compounds or groups of compounds (Wiegand and Perry, 1961; Carlsson and Lindquist, 1963). Estimation of an amine or an amine metabolite in the presence of other related substances is not, however, always satisfactory if the concentration of the substance being measured is low relative to the concentrations of other compounds present in the assay solution. This situation is liable to occur in brain extracts, particularly after treatment of the animal with a drug causing a preferential rise in the concentration of certain of the amines or their metabolites in brain tissue.

#### Statement of the Problem

A method capable of separating and assaying the catecholamines, their precursors and metabolites in the one brain sample would avoid the dangers and limitations attendant upon estimating one compound in the presence of another or others. The least amount



of amine or metabolite detectable in a sample would at the same time be decreased because division of the sample into several portions would no longer be necessary. This section of the thesis describes the development of such a method, which is applicable to brain tissue and can therefore be used in studies on the metabolism of the catecholamines in brain and the effects of drugs thereon.

The possible catecholamines, their precursors and metabolites present in brain are shown in Figure 1. They can be sub-divided into (i) the catecholamines - adrenaline, noradrenaline and dopamine, (ii) the amino acid precursors - tyrosine and dihydroxyphenylalanine (dopa), (iii) the methoxy derivatives of the catecholamines - metanephrine, normetanephrine and methoxydopamine; (iv) the acids - dihydroxymandelic acid and dihydroxyphenylacetic acid with their respective methoxy derivatives - vanillinmandelic acid and homovanillic acid and (v) the alcohols (not shown in Figure 1) which represent an alternative pathway following the action of monoamine oxidase on each of the amines (Rutledge and Jonason, 1967).

This section describes a technique for the separation of (i), (ii), (iii) and (iv) and for the assay of the components of (i), (ii) and (iii) in the same brain sample, with the exclusion of an assay method for tyrosine. Description of the separation and assay



of the acids will be brief, since this work was done, concurrently with work on the amines, by a colleague, Dr Guldberg. In order to limit this study, the alcohols were not investigated.

### Historical Introduction

The literature survey is divided into three parts, corresponding to the three stages involved in determining several catechol derivatives in a tissue. These are:

- (1) extraction of the compounds from the tissue,
- (2) separation of the compounds, one from another,
- (3) estimation of the separated compounds by fluorescence techniques.

These stages are inter-dependent in that selection of a particular technique for any stage has to be made with due consideration of the techniques used for the other two stages. For maximum sensitivity, the most sensitive method of fluorescence assay should be chosen and extraction and separation procedures evolved to allow for the application of this method.

#### (1) Extraction of the Amines and their Metabolites from Tissues

Since the catechols are more stable in an acidic environment, initial homogenisation of the tissue is

always done in acid solution, usually in 0.01N hydrochloric acid or 0.4N perchloric acid. Soft tissues such as brain and adrenal medulla can be readily broken down by homogenisation in a glass homogeniser. The amines and their metabolites may be extracted from the acid homogenate into an organic solvent or the protein may be removed by precipitation and centrifugation to give an aqueous acidic supernatant which contains the amines and their metabolites. Shore and Olin (1958) found that adrenaline and noradrenaline could be extracted into n-butanol from a salt saturated tissue homogenate prepared in 0.01N hydrochloric acid. The amines were back-extracted into acid by shaking the butanol layer with acid and heptane. This technique is not advised for tissues, such as brain, which have a low amine content, since only 65% of the adrenaline and noradrenaline and none of the dopamine (Cass and Spriggs, 1961) is extracted. The alternative method, entailing clarification of the acid homogenate by precipitation of the protein is therefore more usually employed.

Various protein precipitants have been used. Occasionally tissues homogenised in 0.1N sulphuric acid (Laverty and Sharman, 1965) show adequate protein precipitation but usually addition of a protein precipitant such as methanol (Montagu, 1957), ethanol (Crawford and Outschoorn, 1951), equal parts of methanol

and acetone (Hagopian, Dorfman and Gut, 1961), trichloroacetic acid (Sourkes and Drujan, 1957) or perchloric acid (Bertler, Carlsson and Rosengren, 1958) to the acid homogenate, is required. Choice of protein precipitant depends largely on the next step in the procedure. If the extracted material is to be applied to a paper chromatogram, 0.01N hydrochloric acid plus ethanol is useful since it can be readily evaporated in vacuo. The acid precipitants are best removed before any evaporation, since on concentration they give rise to concentrations of hydrogen ion which could destroy small amounts of the amines. In addition, perchloric acid is an oxidising agent and in increased concentration could oxidise the amines (Anton and Sayre, 1962). Perchloric acid can be removed as its relatively insoluble potassium salt (Bertler et al., 1958). Trichloroacetic acid can be extracted into ether but losses of the amines may occur during this procedure (Bertler et al., 1958). Extracts applied to ion exchange or alumina columns must be free of organic solvents to avoid emulsification of lipids extracted into the solvent, by the aqueous phase in the columns. Acid ethanol is therefore not a suitable preliminary to ion exchange or alumina chromatography (Bertler et al., 1958). Perchloric acid extracts are the most widely used for application

to both ion exchange columns and paper chromatograms. Perchloric acid has an advantage over ethanol in that it does not extract lipids (Callingham and Cass, 1963, p. 21). The initial homogenisation in 0.01N hydrochloric acid can be omitted and the tissue homogenised directly in perchloric acid (Bertler et al., 1958).

No quantitative studies comparing methods of amine extraction from tissues could be found in the literature. There are, however, reports on conditions conducive to optimal extraction and stability of the amines. These are:

(i) Extraction of the tissue: The tissue should be extracted immediately after removal from the animal (Vogt, 1954) or immediately frozen solid. Graborits, Chessick and Lal (1966) found there was no loss of noradrenaline from brain after four hours in the deep-freeze, providing the brain was homogenised without thawing.

(ii) Temperature of extraction: The tissue and extracting fluid should be kept thoroughly chilled (Häggendal, 1966; Anton and Sayre, 1962). Precipitation of proteins and clarification of extracts are aided by a low temperature and most workers recommend centrifuging extracts at  $4^{\circ}$  -  $10^{\circ}\text{C}$  (Bertler et al., 1958).

(iii) Number of extractions necessary to remove the amines from the tissue: Bertler et al. (1958)

investigated the efficiency of catecholamine extraction from brain and heart by repeated extraction with three volumes 0.4N perchloric acid per g tissue. They found on average 98% removal in the first two combined extractions, 2% in the third and zero in the fourth. They also found that the amines distributed themselves between the supernatant fluid and the precipitate as though their concentrations in the supernatant and in the water phase of the precipitate were the same. Anton and Sayre (1962) and Gunne (1963) have used this finding to correct for amine loss in the precipitate after extraction of the brain tissue with 6 to 10 volumes of 0.4N perchloric acid. The correction factor with such large volumes of extracting fluid is small and most workers have ignored it without any consideration of the error involved.

(iv) Addition of anti-oxidants: Catecholamines are readily oxidised in dilute solution (von Euler, 1956, p. 11). Reducing agents such as ascorbic acid or sodium metabisulphite are often added during the extraction process. This is possibly an unnecessary precaution, since brain extracts have themselves a stabilising effect on the amines (Vogt, 1954; Shore and Olin, 1958).

(v) Addition of chelating agents: Ethylenediamine tetra-acetic acid (EDTA) is often added during

extraction to protect the amines against attack by metal ions (von Euler and Floding, 1956). In acid solution, heavy metals such as copper (Häggendal, 1963) catalyse oxidation of the catecholamines. The necessity of the addition of EDTA probably depends on the purity of the water supply. Distilled deionised water should not contain any metal ions, but these might originate from materials used during the extraction or separation processes, particularly from alumina (Anton and Sayre, 1962; 1964).

(vi) Effect of pH on the stability of catecholamines in tissue extracts prepared with 0.4N perchloric acid: The pH of these extracts is about 0.5 - 1.0 and under these conditions noradrenaline is stable for one week at 0°C (Bertler et al., 1958). Stability can be prolonged to three months by adjusting the pH to about 3.8 and storing in a deep-freeze (Günne, 1963).

The acid metabolites of the catechol- and methoxyamines are extracted from tissues by the same procedures and with the same precautions as are employed to extract their parent amines. Carlsson and Hillarp (1962) have used 0.4N perchloric acid to extract dihydroxyphenylacetic acid and homovanillic acid from rabbit brain and Hagopian et al. (1961) used hydrochloric acid followed by acetone-methanol to extract both acids and amines from bovine hypothalamus.

The acid metabolites in the extract can be separated from the amines and the amino acids by extraction into ethyl acetate (Carlsson and Hillarp, 1962). Sharman (1963) reported that saturation of the aqueous phase with sodium chloride was essential for quantitative transfer of homovanillic acid into the organic layer. In the few reported experiments on amine and acid levels in the same sample of tissue (Lavery and Sharman, 1965; Juorio and Vogt, 1965), separate aliquots have usually been taken for assay of the amines and the acids, respectively. The effect of the ethyl acetate extraction on the estimation of the amines remaining in the aqueous has not been determined.

## (2) Methods of Separation of the Amines

There are three techniques for separating the catecholamines namely, (i) adsorption onto alumina, (ii) ion exchange chromatography and (iii) paper chromatography.

(i) Adsorption onto alumina. Alumina was introduced as the first specific adsorbent for the catecholamines by Shaw (1938). Unlike ion exchange and paper chromatography, it does not separate the amines one from another. Adsorption onto alumina is specific to the catechol nucleus (Lund, 1949b; von Euler and Orwén, 1955), so that the catecholamines, their catechol



Precursor dopa and catechol metabolites dihydroxyphenyl-acetic acid and dihydroxymandelic acid are all adsorbed. These compounds are eluted simultaneously and may be estimated in the presence of one another by selection of specific methods of assay. Alumina adsorption is, however, more frequently employed as a preliminary purification technique for tissue extracts before separation of the individual catechols by ion exchange or paper chromatography (Whitby, Axelrod and Weil-Malherbe, 1961; Glowinski, Iversen and Axelrod, 1966).

(ii) Ion exchange chromatography. Previous to the introduction of ion exchange resins, von Euler, Hamberg and Hellner (1951) used partition on a starch column to separate adrenaline, noradrenaline and dopamine. Separation by this technique was very slow, since it depended entirely upon non-ionic differences between the partition characteristics of the amines. With ion exchange columns ionic as well as non-ionic differences are operative and separation becomes possible with a much shorter time for elution. Catecholamines are usually adsorbed as their cations, since they are unstable at the alkaline pH necessary for their ionisation as anions. (The pKa for adrenaline ionised as an anion is 10.2 and as cation, 8.7).

The first ion exchangers used for separation of

the catecholamines were the weak cationic resins. These have the disadvantage that they only function at a pH greater than 6, at which pH the catechols are not very stable. Metanephrine and normetanephrine, which are more stable at pH 6 - 6.5 than their parent catechols, were isolated using a weak cationic resin by Pisano (1960) and Smith and Weil-Malherbe (1962).

Strong cationic exchangers have two main advantages over their weak counterparts, (1) they adsorb cations very readily at almost any pH, so that the amines may be applied in acid solution, in which they are more stable and (2) it is possible to elute the adsorbed amines preferentially with different concentrations of acid and thus obtain separation of at least some of the amines without lengthening the column disproportionately.

Ellman (1956) first used a strong cationic resin to adsorb catecholamines at pH 2 from an extract of adrenal medulla. Häggendal (1962a) found that complete separation of adrenaline, noradrenaline, dopamine, metanephrine, normetanephrine and methoxydopamine was possible using 1N hydrochloric acid as the eluting fluid only if the cationic resin column was 105 cm long. Varying degrees of separation were achieved with shorter columns. Elution from long columns took several hours during which time there was considerable

amine loss, which could partly be avoided by the addition of ascorbic acid and EDTA. Large amounts of acid were required to develop these long columns and as a result the separated amines were obtained in large volumes of eluant which considerably reduced the sensitivity of amine assay. Despite thorough washing, these columns released a material during acid elution which, upon treatment with potassium ferricyanide, gave rise to a fluorescence similar to that derived from the catecholamines, thus further reducing the sensitivity of amine assay. For these reasons, Häggendal's method for separating all the amines at once has not been generally adopted. Using much shorter columns of a strong cationic resin which therefore required much less eluting fluid, Bertler et al. (1958) obtained almost quantitative separation of adrenaline and noradrenaline from dopamine by eluting the former two amines in 1N hydrochloric acid followed by the latter amine in 2N hydrochloric acid. Although there is little published evidence, it seems likely that this is the limit of amine separation possible with short columns. Glowinski, Iversen and Axelrod (1966) and Iversen, Glowinski and Axelrod (1966) have used such columns in conjunction with alumina adsorption, to separate radio-active dopa, noradrenaline, dopamine and the methoxyamines. The methoxyamines were not

separated from one another using this technique.

Wright (1958) and Takahashi and Fitzpatrick (1964) have used anionic resins to remove catecholamines and dopa from urine. Oxidation at the alkaline pH necessary for amine adsorption onto the anionic resin was prevented by complexing the catechols with borate before applying them to the column. Recoveries of adrenaline and noradrenaline were poor. Strong cationic resins remain the exchangers of choice.

(iii) Paper chromatography. James (1948) first demonstrated that adrenaline and noradrenaline could be separated on a paper chromatogram using phenol. The only other solvent systems known to separate the amines are n-butanol:acetic acid:water (4:1:5) or n-butanol equilibrated with N hydrochloric acid (Shepherd and West, 1953; McGeer and Clark, 1964). Phenol gives the better separation and has been preferred for the chromatographic separation of adrenaline and noradrenaline from tissue extracts. Vogt (1959) showed dopamine and dopa had almost the same R<sub>f</sub>s in phenol containing 1% (v/w) 0.1N hydrochloric acid as adrenaline and noradrenaline, respectively. When biological methods of assay were used (Holton, 1949; Crawford, 1951; Crawford and Outschoorn, 1951) these compounds did not contribute to the adrenaline and noradrenaline estimates. They could, however,

contribute to estimates obtained by chemical assay. One disadvantage of phenol chromatography is that phenol is leached out of the chromatogram into the paper eluates from which it must be removed since it interferes with both biological and chemical methods of assay. For quantitative recovery, suitable precautions such as development of the chromatogram in an atmosphere of an inert gas and the use of freshly distilled phenol must be taken to guard against amine oxidation during chromatography.

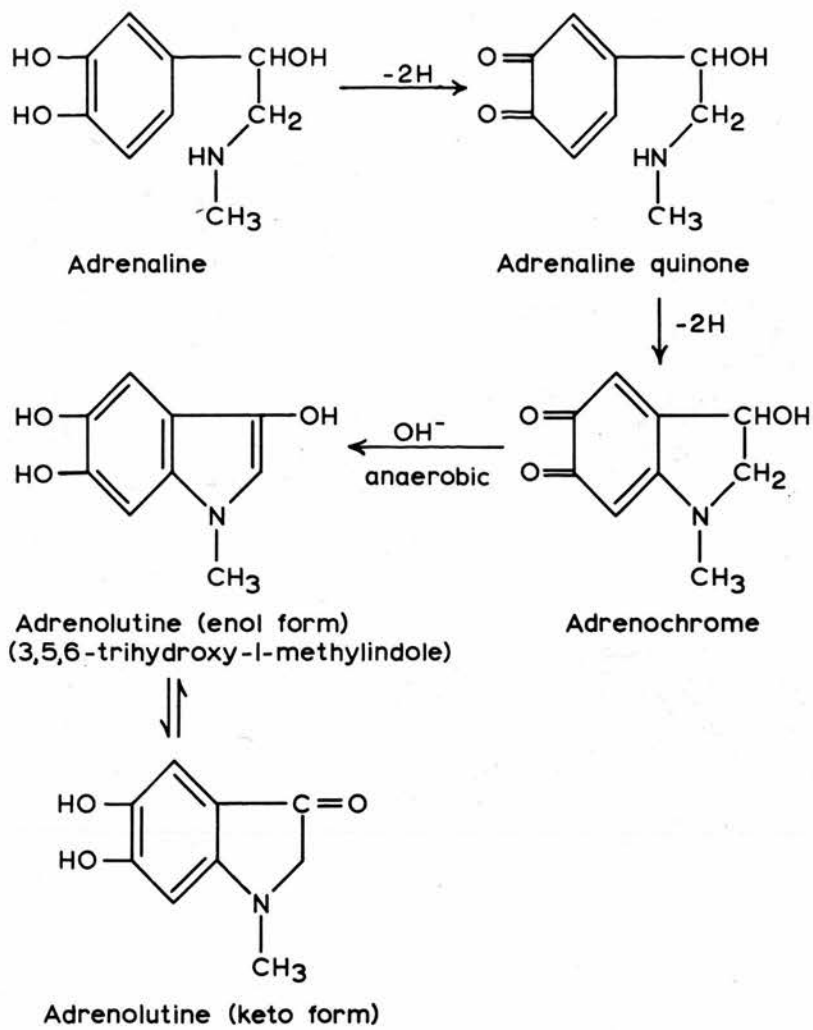
Weiss and Rossi (1962) separated adrenaline, noradrenaline, dopamine and dopa using descending chromatography in phenol:hydrochloric acid, drying the chromatogram and then developing it, in the opposite direction, in n-butanol saturated with 1N hydrochloric acid. Their technique was tested only qualitatively but it represents one of the few attempts to improve the resolution of the separation of the amines in phenol:hydrochloric acid.

### (3) Fluorescence Methods of Assay

Catecholamines fluoresce in their native form. The fluorescence is, however, weak and since it is due to their phenolic structure, not specific (Udenfriend, 1962, p. 140). There are two methods currently

available for producing highly fluorescent derivatives from the catecholamines. The older and more specific is the 'trihydroxyindole' method; the other more recent and less specific method involves coupling with ethylenediamine. The sensitivities of the amine assays by these two methods are approximately the same.

(i) The trihydroxyindole method. Loew (1918) first observed that the oxidation products of adrenaline fluoresced in alkaline solution. Gaddum and Schild (1934) and Hueber (1940) used this yellow-green fluorescence to assay adrenaline but commented on its evanescence. Stabilisation of the fluorescence by addition of ascorbic acid along with the alkali (Ehrlén, 1948; Lund, 1949b) allowed the reaction to be used for routine adrenaline assays. The chemical constitution of adrenolutine, the adrenaline fluorophor, is not known with certainty, but is most likely to be a trihydroxyindole, specifically the 1-methyl-5,6-dihydroxy derivative of indoxyl which itself possesses a strong yellow fluorescence (Lund, 1949a). In a following paper, Lund (1950) reported formation of a similar fluorophor from noradrenaline if oxidation was carried out at pH 6.5; the pH is much less critical for oxidation of adrenaline. In order to achieve consistent quantitative production of fluorescence, oxidation must be allowed to proceed for a controlled

Figure 2FORMATION OF ADRENOLUTINE FROM ADRENALINE



of period/time (Lund, 1949b). Various modifications to Lund's original method have been introduced, in particular the use of potassium ferricyanide (von Euler and Floding, 1956) or iodine (Drujan, Sourkes, Layne and Murphy, 1959) as the oxidising agent instead of manganese dioxide, which was used by Lund. The mechanism of the reaction has been explained by Lund (1949a) and Harley-Mason (1950) as follows: The amine is oxidised in acid solution for a period of time sufficient to allow the chrome, but not the oxidation product from the chrome (melanin), to form. The chrome is then re-arranged to the fluorescent lutine derivative by alkali in the presence of a reducing agent to prevent oxidation of the lutine (Figure 2).

Formation of the dopamine fluorophor occurs much less readily than that of the adrenaline or noradrenaline fluorophors. A strong oxidising agent such as periodate (Anton and Sayre, 1964) or iodine is required and the slow development of the fluorophor (Bischoff and Torres, 1962) can be speeded up by U-V irradiation (Carlsson and Waldeck, 1958) or heating (Udenfriend, 1962, p. 137). The fluorescence derived from dopamine is intensified and its excitation and fluorescence maxima changed from those of the fluorophors from adrenaline and noradrenaline if the solution is acidified. Bu'lock (1960) suggested that although

the alkaline fluorescence might be due to the dihydroxy-indole derivative of dopamine, the acid fluorescence was more likely to originate from the tetrahydroxy-di indole derivative. Methoxydopamine gives rise to a fluorescence which has the same characteristics as the dopamine fluorescence but is ten times less intense (Carlsson and Waldeck, 1964).

Metanephrine and normetanephrine are resistant to oxidation by potassium ferricyanide, unless zinc sulphate is present as a catalyst (Brunjes, Wybenga and Johns, 1964). Alternatively, as in the case of dopamine and methoxydopamine, a stronger oxidising agent such as periodate (Taniguchi, Kakimoto and Armstrong, 1964) or iodine (Bertler et al., 1959b) may be used. Since the fluorophors from normetanephrine and nor-adrenaline fluoresce maximally at about 400/500 m $\mu$  and those from metanephrine and adrenaline at about 410/520 m $\mu$ , it would appear that the fluorophors and presumably their mechanism of formation, are the same in each case. Due to their identical fluorescence characteristics, it is not possible to estimate a catecholamine and its methoxy derivative in a single portion of the sample. Estimation can only be achieved by dividing the sample into two and estimating in one portion the catecholamine plus the methoxyamine and in the other portion the catecholamine alone. In order

to avoid dividing the sample with concomitant loss of sensitivity, a point of particular importance having regard to the low amounts of methoxyamines present in brain tissue (Carlsson and Lindquist, 1962; Carlsson and Waldeck, 1964), it is necessary to separate each of the catechols from their methoxy derivatives previous to assay.

Adrenaline and noradrenaline have been estimated in the same sample, either by utilising the slight difference in the wavelengths for maximal excitation and fluorescence of their fluorophors (Price and Price, 1957; Vendsalu, 1960) or by utilising the fact that whereas both amines are oxidised at pH 6.5, only adrenaline and 5 - 7% of the noradrenaline, is oxidised at pH 3.5 (Lund, 1950; de Schaepdryver, 1958).

Dopa can be assayed in the same way as adrenaline and noradrenaline (Bertler et al., 1958).

With the exception of the methoxyamines, all the discussed compounds have the structure considered essential for the trihydroxyindole reaction, namely, a catechol nucleus with a two carbon side chain ending in an amino nitrogen and at least one hydrogen atom attached to each of the side chain carbon atoms (Lund, 1949b).

(ii) The ethylenediamine condensation method.

Natelson, Lugovoy and Pincus (1949) first described the reaction of ethylenediamine with adrenaline to form a fluorophor which was stable and extractable into aliphatic alcohols. Weil-Malherbe and Bone investigated the reactions of catechols with ethylenediamine in more detail and described application of this reaction to the estimation of adrenaline (1952) and noradrenaline and dopamine (1957). Weil-Malherbe and Bone (1952) heated adrenaline at  $50^{\circ}$  for 20 minutes with an aqueous mixture (pH about 10) of ethylenediamine and ethylenediamine dihydrochloride and extracted the fluorophor into isobutanol. Apart from the use of hydrochloric acid (Lavery and Sharman, 1965) or ammonium chloride (Kägi, Burger and Giger, 1957) instead of ethylenediamine dihydrochloride, there have been no worthwhile amendments to this technique. Unlike the trihydroxyindole method, this one set of reaction conditions produces fluorophors from all compounds capable of coupling with ethylenediamine.

Natelson et al. (1949) and Weil-Malherbe (1952) described the reaction with adrenaline as involving oxidation, in alkaline solution, to the quinone, adrenochrome, and subsequent coupling with ethylenediamine. The reaction is not limited to the catecholamines but is given by all compounds having a catechol



nucleus. The fluorophors from dihydroxymandelic acid, adrenaline, noradrenaline and dopamine are extractable from the reaction solution into isobutanol but those from dopa and dihydroxyphenylacetic acid are only extracted to a slight extent, suggesting they retain their carboxyl groups after condensation (Weil-Malherbe, 1959). Adrenaline is considered to produce only one fluorophor, dihydro-3-hydroxy-1-methylpyrrolo-(4:5-g)-quinoxaline, by coupling of one molecule of ethylenediamine onto the quinonoid oxygens of adrenochrome (Harley-Mason and Laird, 1958). Noradrenaline gives a mixture of fluorophors. The main fluorophor is believed to be 1,2,3,4-tetrahydro-1,4,5,8-tetra-aza-anthracene, which is also obtained from condensation of catechol with ethylenediamine (Weil-Malherbe, 1960) (Figure 3). The structure of the dopamine fluorophor has not been worked out apart from the fact that the side chain is not eliminated during the reaction of ethylenediamine with dopamine (Weil-Malherbe, 1959).

Certain of these fluorophors, particularly those from noradrenaline, are light sensitive. Aronow and Howard (1955) suggested working in a red-illuminated room to protect the light sensitive fluorescence. However, loss of the light sensitive component leaves a fluorescence which is stable and perfectly adequate for assay purposes. Weil-Malherbe (1959) and Goldfien (1961) concluded that greater uniformity was achieved



by allowing the light sensitive fluorescence to fade completely than by attempting with varying success to preserve it.

### Fluorescence and its Measurement

Fluorescence occurs when a molecule, after absorbing light at a particular wavelength, emits light of a different wavelength. The wavelengths of maximum absorption, or excitation and of maximum emission, or fluorescence, are characteristic of a given compound and may be used as an aid to its identification. Fluorescence can only be used for quantitative purposes in very dilute solution, in which there is a linear relationship between fluorescence and concentration, if the wavelength and intensity of the exciting light is kept constant.

### Instrumentation

Spectrophotofluorimeters (SPFs) are instruments specially designed for the detection and measurement of fluorescence. The basic components of these instruments are (i) a light source, (ii) two light filtering systems and (iii) a light detection apparatus. Two SPFs, namely the 'Farrand' and the 'Aminco-Bowman' were used in the studies reported in this thesis.

(i) The light source is usually a Xenon arc lamp which is capable of delivering high intensity monochromatic light throughout the U-V and visible spectrum.

(ii) The grating monochromators are used to select the excitation wavelength and to select fluorescent light of a particular wavelength coming from the sample. The excitation spectrum or scan of a compound can be obtained by setting the fluorescence monochromator at a wavelength close to that of maximum fluorescence of the compound and recording the relative fluorescence intensity while exciting the solution containing the compound with light of shorter wavelengths, starting at 200 mμ. The instruments are designed so that this can be done automatically. In a similar manner it is possible to obtain the fluorescence spectrum, by setting the excitation monochromator at the wavelength for maximal excitation of the fluorescence and continuously changing the wavelength of the light transmitted through the analysing monochromator to the detector. The band widths of the light activating the solution and of the light reaching the detector can be varied by the insertion of slits of different widths between the monochromators and the sample. Reducing the band width of the existing and/or fluorescent light increases resolution at the cost of diminished fluorescence due to decreased light intensity. Wide band widths were



used in the assays described in this thesis, in order to obtain maximum excitation and hence maximum fluorescence, from the very small amounts of material, often less than 50  $\mu\text{g}/\text{ml}$ , present in the solution in the cuvette. Excitation (x) and fluorescence (y) wavelengths used in any procedure in this thesis are uncorrected for instrumental variation and are written thus: x/y  $\mu\text{u}$ .

(iii) The emitted fluorescent light is detected by means of a photomultiplier tube. In the following investigations IP 28 tubes, which are more sensitive to U-V light than IP 21 tubes, were used in both instruments. Renewal of a worn-out tube may result in altered and possibly reduced readings for the fluorescence of standard solutions because the sensitivity of individual tubes can vary widely (Aminco-Bowman catalogue, 1963, p. 18).

Practical considerations for the preparation of fluorescent solutions. Great care is necessary when measuring catecholamines by fluorimetry due to the susceptibility - which is much increased at concentrations as low as those encountered in the work described in this thesis - of the amines and their fluorophors to pH (Williams, 1959), temperature (Udenfriend, 1962, p. 106), various ions (Anton and Sayre, 1966) and other, often unknown, factors.

Fluorescence assays are notoriously fickle and can be influenced by light and in particular by substances, often unidentified (Lavery and Sharman, 1965) present in the water supply used to make up the reagents. We found distilled water passed through a mixed-bed ion exchange resin was satisfactory. All-glass apparatus, cleaned in chromic acid, was used. Thorough rinsing in tap water, followed by distilled water, was necessary to remove the chromic acid, which itself has a fluorescence (Udenfriend, 1962, p. 99). Polythene apparatus is not advised as it may adsorb traces of the amines and/or their fluorophors.

In order to obtain maximum specificity and sensitivity in any assay, the fluorescence of the reagents must be as low as possible. Analytical grade chemicals and deionised distilled water were used to this end. The light scatter peak was minimised by using absolutely clear reagent solutions. Reagents should be renewed frequently, especially buffer solutions, which tend to form fine deposits on standing. Scattered light reveals itself on the fluorescence spectrum (p. 26) by an emission peak, distinguishable from a fluorescence peak by the fact that its wavelength of maximum emission is identical with the excitation wavelength. If the wavelengths of maximum excitation and fluorescence of a compound are close

together, the light scatter peak may not be completely separated from the fluorescence peak and may contribute to the sample reading, which is usually taken at the wavelengths of maximum excitation and emission of the compound being estimated.

#### Preparation of Blanks

In any assay method it is necessary to determine the contribution made to the measurement, by substances present other than that being measured so that this contribution by the 'blank' may be allowed for in the estimation. In pure solution, the fluorescence of the blank consists of the fluorescence resulting from admixture of all the reagents used during the assay. This blank is referred to as the 'reagent' blank. When a substance is estimated in the presence of a tissue extract, its blank must also include the fluorescence originating from components of the extract or from the reaction of these components with any of the assay reagents. Such a blank is called a 'tissue' or 'extract' blank. Preparation of a representative tissue blank is a major problem in the use of fluorimetry for the assay of substances in tissues. In the trihydroxyindole method for the assay of catecholamines, 'faded' blanks, in which the catecholamine fluorophor is allowed to decompose before adding the

stabilising, reducing agent, are frequently used as tissue blanks. Other methods of forming tissue blanks include omission of a reagent vital to the production of the fluorophor or addition of the reagents in an order different from that required for fluorophor formation. All these techniques have their own disadvantages and the method of determining the blank value must be tailored to suit each individual assay.

### Standards

Known amounts of the substance being assayed should always be included with each batch of samples, since the fluorescence intensity derived from the same amount of substance can vary on different occasions. The amount of fluorescence derived from a standard in the presence of a tissue extract may be different from that produced from the same standard in pure solution. This is because materials present in an extract can sometimes influence the fluorescence, usually reducing or 'quenching' it. Measurement of such interference is achieved by processing an aliquot of the tissue extract to which the standard has been added, in parallel with the sample. The standard added to the tissue extract is referred to as the 'internal standard'. Comparison of the fluorescence from this internal standard with that from the same

standard in pure solution (the 'aqueous' standard) gives a measure of the extent of interference caused by the tissue extract and the sample estimates can then be corrected accordingly.

### Sensitivity of an Assay

The sensitivity of an assay i.e. the smallest amount of substance that can be measured depends upon (i) the fluorescence yield from the substance, (ii) the size of the blank and (iii) the degree of quenching, if any. In this thesis, the sensitivity of an assay is assessed, arbitrarily, as that amount of substance which produces a fluorescence reading equal in size to that produced by the appropriate blank at the same wavelengths of excitation and emission. It is, however, possible to measure, albeit with diminished accuracy, amounts of a substance giving rise to a fluorescence relatively less than this. The lowest limit of estimation of a substance with any degree of confidence has been taken as that amount of substance giving rise to a fluorescence intensity equal to half that of the blank. In subsequent discussions this limit has been referred to as the 'level of detection'.

## REAGENTS

Unless otherwise stated in the text, all reagents used were of analytical grade.

Distilled de-ionised water (p.28 ) was used for all procedures described in this thesis and for preparing all solutions.

Hydrochloric acid: BDH Micro-analytical reagent.

Ethylenediamine: 1:2 diaminoethane (BDH, Laboratory Reagent), was distilled three times, only the middle one third fraction being collected from each distillation. The re-distilled ethylenediamine was stored in a brown-glass bottle and was used for not more than 2 weeks.

Potassium ferricyanide: Analar, recrystallised from water.

Ethyl acetate: Following on Farrand's (1966) studies on the adverse effect of ethyl acetate which had been exposed to light and air for periods of 2 - 48 hr., on the determination of vanillin-mandelic acid, ethyl acetate (Analar) was obtained in 500 ml brown-glass bottles. These bottles were stored in a dark cupboard and a fresh bottle used for extractions in each experiment.

Dichloromethane: Dichloromethane (BDH, Laboratory Reagent) was distilled and that fraction boiling at 40°C used for extractions.

Methanol: Methanol (Analar) was distilled once before use.

Isobutanol: iso-Butyl Alcohol FT, 'for the fluorimetric determination of vitamin B<sub>1</sub>' (BDH, Laboratory Reagent) was used. It was purchased in 500 ml bottles and the contents used within two weeks of opening a bottle.

Toluene: Toluene (Analar) was distilled once before use.

Ethanol: Ethanol was refluxed for 2 hr. with 20 g sodium hydroxide per 400 ml ethanol and then distilled twice.

Starch Glycerin Paste: 20 g starch and 70 g glycerin was heated until it was liquid and just started to thicken. It was then removed from the heat and stirred continuously until thick.

#### Drugs

Adrenaline: (-)-Adrenaline bitartrate, Burroughs Wellcome.

Noradrenaline: (-)-Noradrenaline bitartrate, Bayer Ltd..

Dopamine: 3-Hydroxytyramine hydrochloride, Koch-Light Lab..



Dopa: DL-3,4-Dihydroxyphenylalanine, Koch-Light Lab..

Metanephrine: (+)-Metanephrine hydrochloride, Calbiochem. B grade.

Normetanephrine: (+)-Normetanephrine hydrochloride, Calbiochem, B grade.

Methoxydopamine: 3-Methoxy-4-hydroxyphenylethylamine, Calbiochem. B grade.

Tyramine: Tyramine hydrochloride, BDH Laboratory Chemicals.

5-Hydroxytryptamine: Serotonin creatinine sulphate, Koch-Light Lab..

Octopamine: (+)-Octopamine hydrochloride, K. and K. Lab. Inc..

Stock solutions of 10 mg base/ml of the amines and 1 mg/ml of dopa in 0.01N hydrochloric acid were stored at  $-4^{\circ}\text{C}$  and renewed every 6 weeks. Dilutions of 1 - 10  $\mu\text{g/ml}$  in 0.01N hydrochloric acid were prepared fresh from these stock solutions.

#### SUB-SECTION 1

Estimation of noradrenaline, adrenaline, dopamine, normetanephrine, metanephrine and dopa in rat brain. Determination after extraction and subsequent separation by paper chromatography using a phenol : hydrochloric acid solvent system.

Investigations in this department had indicated that metanephrine, normetanephrine and methoxydopamine, in addition to adrenaline, noradrenaline and dopamine as reported by James (1948) and Crawford (1951) (p. 15 ), could be separated by paper chromatography using phenol containing hydrochloric acid.

In 1960, Sharman, in this department, found that tryptophan, tryptamine and 5-hydroxytryptamine, which in large concentration might interfere in the catecholamine assays, could be separated from the catechols by ascending paper chromatography in 0.1N hydrochloric acid saturated with sodium chloride. Since we intended to study the effect of tryptophan loading on the catechol pathway, this initial separation of the catechol from the indole group was thought advisable before separation of the individual catechols.

A method for the estimation of dopa, noradrenaline, adrenaline, dopamine, normetanephrine and metanephrine in rat brain, which was based on these techniques, is described in this sub-section. Methoxydopamine was excluded because at the time of this investigation there was no way of assaying it.

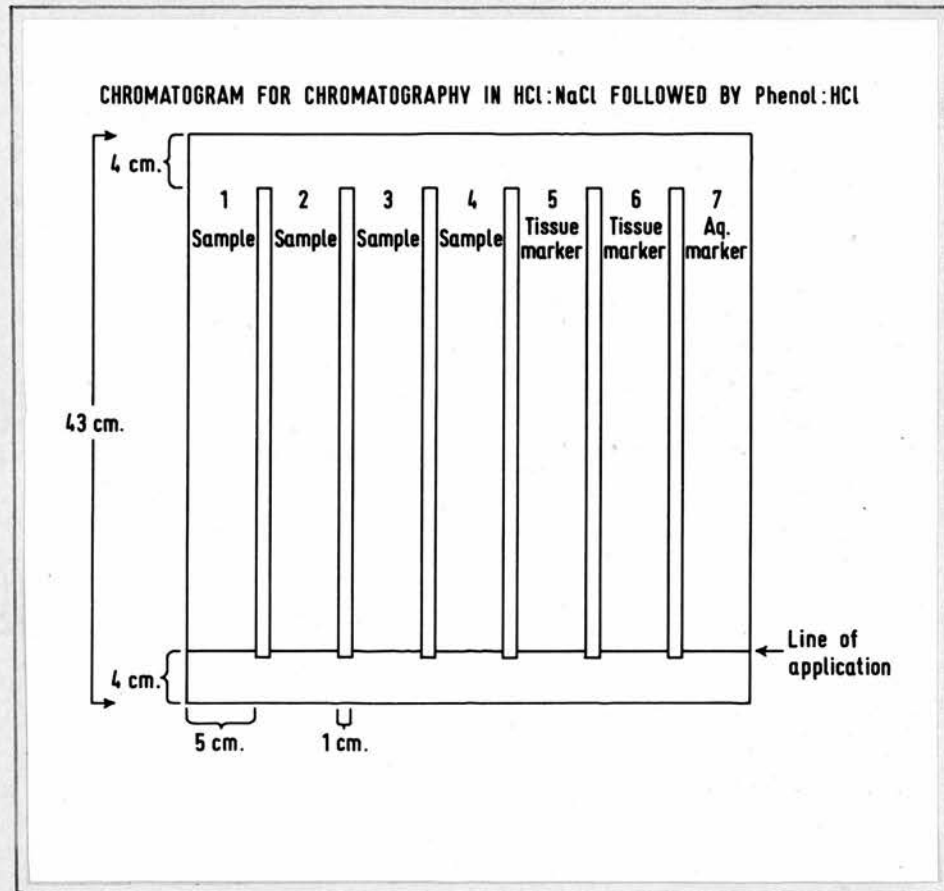
## METHODS

Extraction and Separation of Amines and Dopa  
from Rat Brain

Three or four rats were killed by decapitation and their brains rapidly removed and pooled (minus cerebellums) to give a total weight of 4 - 6 g brain tissue. The brains were homogenised in an all-glass homogeniser for 4 min. in 3 vol. 0.4N perchloric acid at 4°C. The homogenate, contained in a measuring cylinder, was placed in the deep-freeze (-20°C) for 30 - 60 min.. The homogenate was then allowed to thaw and mixed by carefully inverting the measuring cylinder 3 or 4 times. Using a 5 ml safety pipette, six portions of 3.0 to 4.0 ml each were transferred into separate centrifuge tubes. Known amounts of the amines and dopa, contained in 0.10 ml 0.01N hydrochloric acid, were added to two of these portions to determine the recoveries of the amines and dopa through the procedure; these portions will be referred to as procedure recoveries. The six samples of homogenate were centrifuged and the supernatants removed. The pH of each supernatant was adjusted to about 4, using bromophenol blue paper as external indicator and adding dropwise 5N potassium carbonate followed by 1N when the end-point was near. The mixtures were kept

kept at  $-20^{\circ}\text{C}$  for 30 min. (or left overnight at  $-20^{\circ}\text{C}$  if more convenient), then thawed and centrifuged for 10 min. at 3020 g at  $4^{\circ}\text{C}$ . The precipitates were washed with 0.5 ml ice-cold water and re-centrifuged. The bulked supernatants were evaporated under reduced pressure at  $45 - 50^{\circ}\text{C}$  (external temperature) to approximately 0.5 ml and remaining traces of potassium perchlorate removed by adding 5 ml ethanol, chilling and centrifuging. The potassium perchlorate precipitates were washed with 1 ml ethanol and the ethanol supernatants combined and evaporated under reduced pressure at  $45 - 50^{\circ}\text{C}$  to damp dryness. 100  $\mu\text{g}$  adrenaline, noradrenaline, dopamine, dopa, 5-hydroxytryptamine, tryptamine and tryptophan and 200  $\mu\text{g}$  metanephrine and normetanephrine were added, in 0.10 ml 0.01N hydrochloric acid, to two of the four samples. These samples will be referred to as the tissue markers and they were used to determine the Rf of each amine and dopa on the chromatograms in the presence of the tissue extract. They contained amounts of the amines and amino acids sufficiently great to be visualised as coloured bands when streaked across 5 cm wide sodium chloride impregnated filter paper and treated with certain reagents (p. 41 ).

The extracts were taken up in 0.1 to 0.2 ml

Figure 4

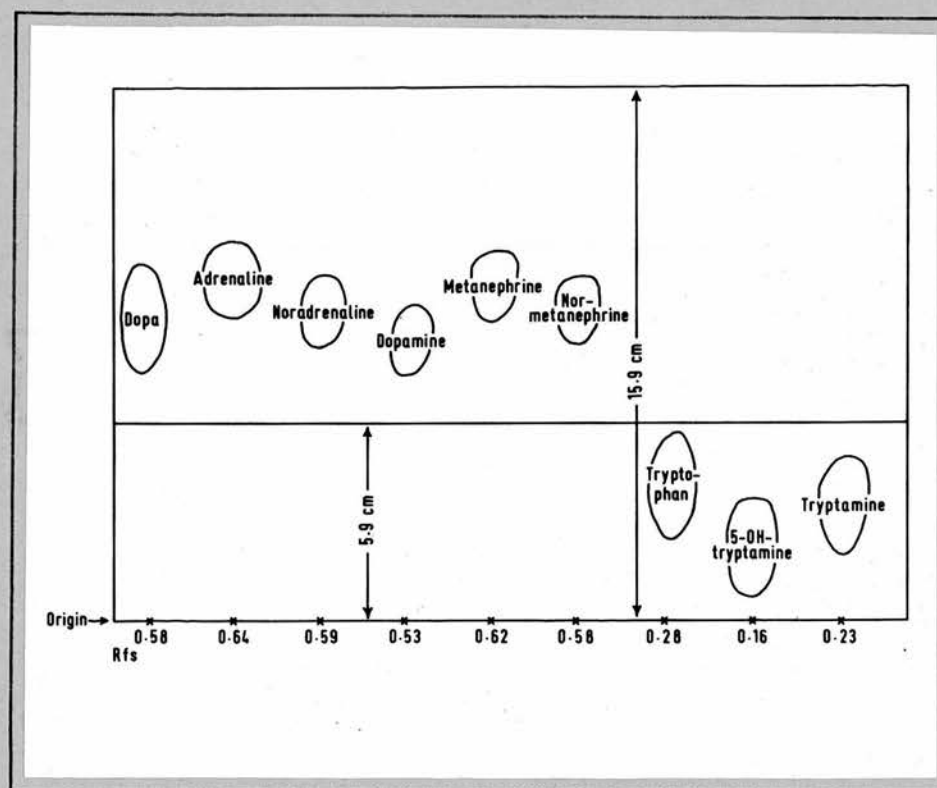
80% (v/v) methanol containing 25 mg % (w/v) ascorbic acid. They were applied across 5 cm wide strips of Whatman no. 1 chromatography paper which had been washed by descending chromatography for 24 hr. in 0.01N hydrochloric acid and dried in air several hours before use. Care was taken to ensure that the extract did not diffuse more than 0.5 cm from either side of the line of application. A stream of nitrogen was projected on to the paper at the site of application of the solution in order to speed up the evaporation of the solvent and to decrease the possibility of losses by oxidation. About 20  $\mu$ g adrenaline, noradrenaline, dopamine and dopa and about 40  $\mu$ g metanephrine and normetanephrine were applied as discrete spots of not more than 0.3 cm diameter, across a seventh 5 cm wide paper strip and 100  $\mu$ g tryptophan streaked over these spots, once they had dried. After chromatography and treatment with the appropriate reagents the positions of the spots on this strip, known as the aqueous marker, served to identify the separated compounds, visible as coloured bands on the tissue marker strips. The seven chromatogram strips required in one experiment, together with the two 4 cm wide cross-pieces of paper holding the strips together, were cut out of one piece of filter paper (Figure 4 ). The chromatograms



Figure 5

Chromatogram of the Catecholamines, Methoxyamines, Dopa and the Indoles run for 2 hr. in an ascending solution of 0.025N hydrochloric acid saturated with sodium chloride.

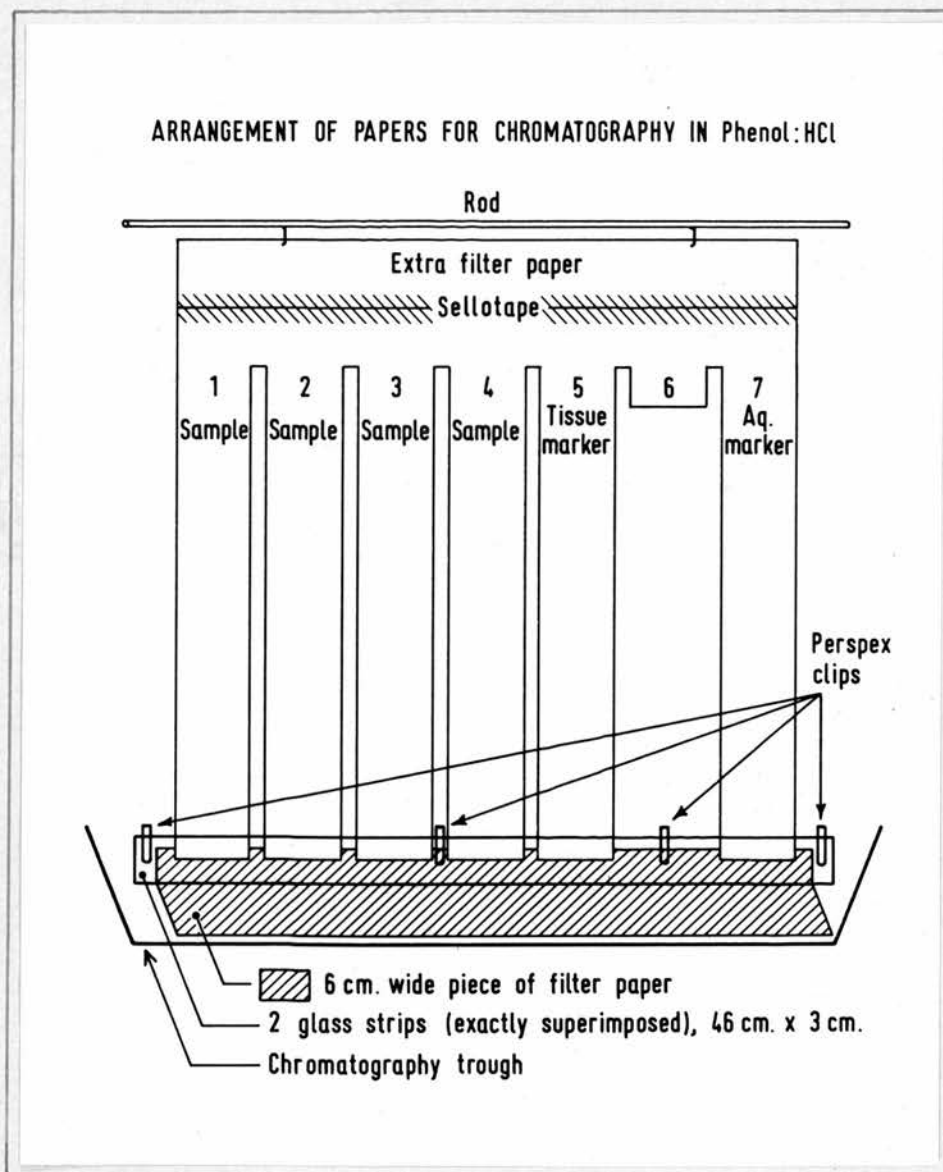
Catechols and methoxyamines visualised with alkaline ferricyanide. Indoles visualised with dimethylaminobenzaldehyde.



were developed in an atmosphere of nitrogen at 18 - 20°C in an ascending saturated solution of sodium chloride containing 0.025N hydrochloric acid, for 2 hr., during which time the solvent front reached 15 - 16 cm from the origin. The concentration of hydrochloric acid was not critical; all that was required was a sufficiently acid medium to prevent oxidation of the catechols. The chromatograms were then dried in a glass tank through which a brisk stream of nitrogen was passed. One of the two marker chromatograms was cut out and divided longitudinally to give two narrow strips. On one strip the catechols were visualised as pink spots showing a yellow-green fluorescence in U-V light when the strip was sprayed with a solution of 0.5 g potassium ferricyanide in 100 ml 0.1M disodium hydrogen phosphate. On the other strip the indoles were visualised as purplish blue areas on treatment of the strip with Ehrlich's reagent (0.5 g dimethylaminobenzaldehyde dissolved in a mixture of 42 ml acetone and 3 ml conc. hydrochloric acid). A typical chromatogram of catechols and indoles run under these conditions is shown in Figure 5 .

Extracts of rat brain did not affect the catechol-indole separation. The indole-bearing regions of the other chromatograms were removed as determined from the

Figure 6



marker chromatogram. Since there was only a 1 - 2 cm separation of the catechols from the indoles, there remained an insufficient depth of amine-free paper to dip into the phenol solvent for further development of the chromatograms. Therefore an additional 6 cm piece of Whatman no. 1 paper was attached to the foot of the chromatograms. At the junctures with the chromatograms there was an overlap of 0.5 cm, contact being maintained by sandwiching the papers between two 3 cm deep strips of glass held together at their extreme ends and between the chromatograms, by perspex clips (Figure 6 ). After positioning the chromatograms and their attachments in the chromatography tank, the intermediate clips ( in Figure 6 ) were removed to allow for easier flow of the solvent. The chromatogram was developed in an ascending solution of phenol containing 15% (v/w) 0.025N hydrochloric acid overnight (about 20 hr.), <sup>in an atmosphere of nitrogen,</sup> between two filter paper curtains saturated with phenol solvent. Fresh phenol redistilled from zinc dust was used for each run (Crawford and Outschoorn, 1951). The temperature during both chromatographic separations was controlled at 18 - 20°C by keeping the chromatography tanks inside a hot-box heated by two electric lamps. Temperatures of over 20°C tended to give poor separations of the

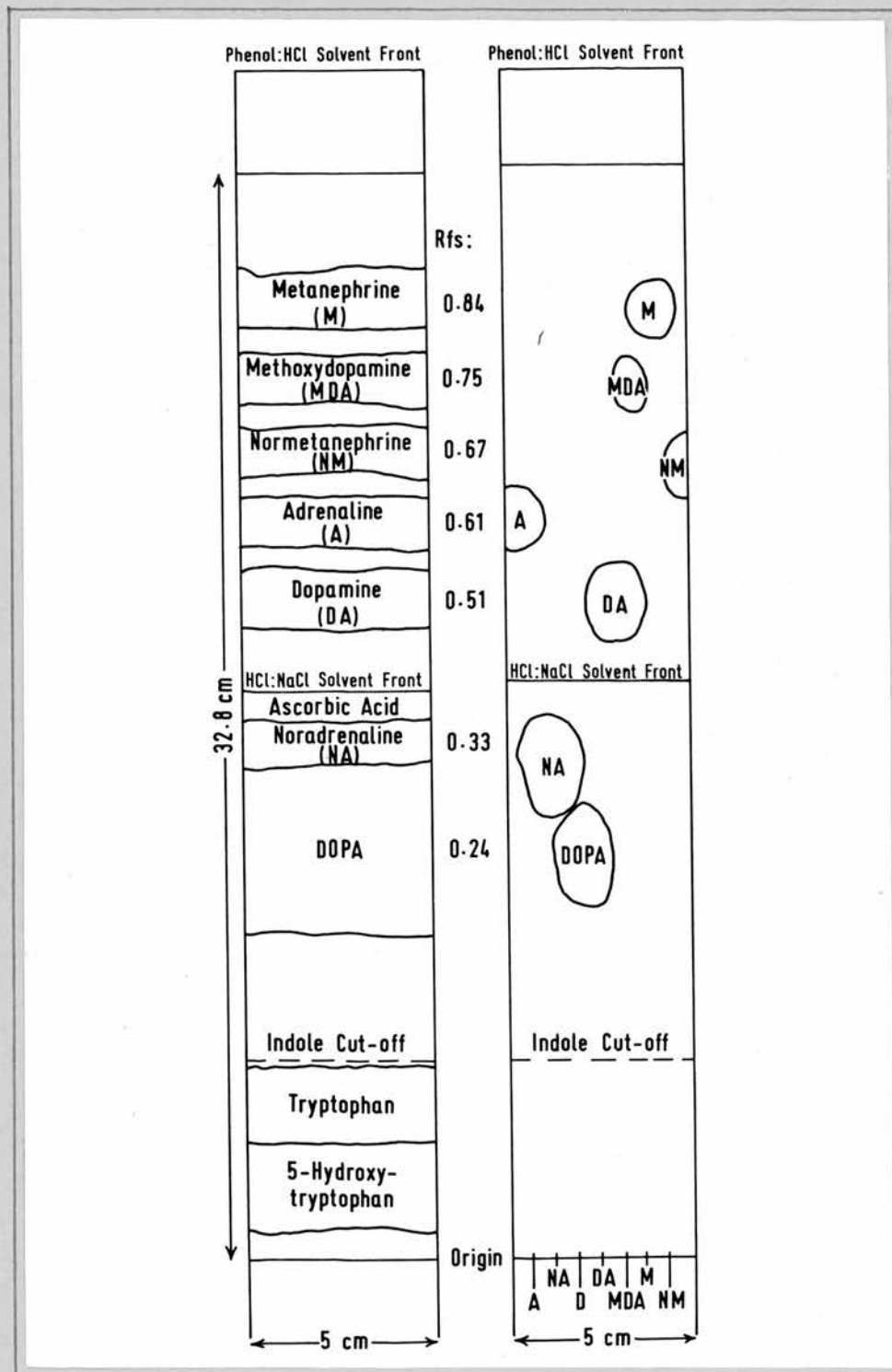
Figure 7

Chromatogram of Catecholamines, Methoxyamines, Dopa and Indoles run for 2 hr. in 0.025N hydrochloric acid saturated with sodium chloride, followed by Chromatography of Catecholamines, Methoxyamines and Dopa, after Indoles had been cut off, for 20 hr. in phenol: hydrochloric acid.

Temp. of run = 15.5 - 18.5°C

Catechols and methoxyamines visualised with alkaline ferricyanide.

Indoles visualised with dimethylaminobenzaldehyde.



indoles from the catechol derivatives and the methoxyamines and of dopamine from noradrenaline. Temperatures below 18°C necessitated increasing the duration of chromatography to more than 20 hr. to achieve the same distance of solvent flow and the same amine separation that was achieved by chromatography at 20°C for 20 hr..

Each chromatogram strip was washed thoroughly in two fresh portions of benzene to remove as much phenol as possible (Crawford and Outschoorn, 1951). The benzene remaining on the paper was removed by hanging the paper strip in a tank through which flowed a brisk stream of nitrogen. The chromatograms carrying the marker amounts of the amines and dopa were sprayed with the alkaline ferricyanide reagent (p. 41 ). Dopa and the catechol amines were satisfactorily visualised by this treatment (p. 41 ) but additional treatment with alkali (spraying with 5M sodium carbonate) was necessary to visualise the methoxyamines by their deep blue fluorescence in U-V light. Sometimes their positions were visible in normal lighting, as uncoloured regions against the pale green background produced by the ferricyanide reagent. Figure 7 is a copy of a typical chromatogram of a mixture of catechols and indoles run in 0.025N hydrochloric acid:



sodium chloride for 2 hr. followed, after cutting off the indole strip, by phenol:hydrochloric acid for 20 hr. at  $15.5 - 18.5^{\circ}\text{C}$ . Methoxydopamine was also included in this chromatogram. Marker amounts of the catechols (100 - 200  $\mu\text{g}$ ) when put through the extraction procedure or when applied to the chromatogram origin together with an extract prepared from an amount of rat brain equal to that being assayed, were, at most, only slightly retarded. On occasion, the upper and lower boundaries of the bands of visualised markers, in particular adrenaline and dopamine, were rather wavy. This was probably due to the difficulty in getting rid of all traces of potassium perchlorate before chromatography and the ensuing difficulty of applying a salt-laden extract evenly to the origin of the chromatogram.

#### Elution of Separated Amines for Estimation

From the sample and procedure recovery chromatograms, the sections bearing the separated amines, as located in the marker chromatogram, were cut out and each was eluted by immersion in 6.0 ml 0.01N hydrochloric acid in glass stoppered test-tubes, for 60 min. at  $4^{\circ}\text{C}$ . The piece of paper was then carefully removed by means of a glass rod. Since noradrenaline and dopa



were not completely separated on the chromatograms they were eluted together. Under favourable conditions adrenaline and dopamine were completely separated and could be eluted independently but more often they had to be eluted together. Metanephrine and normetanephrine were eluted separately, although the separation of normetanephrine from adrenaline was not always very satisfactory.

The paper eluates were stored for up to ten days in the deep-freeze before assay. The amines were assayed as described below.

#### Fluorimetric Estimation of the Amines and Dopa

The methodology was examined using solutions of the appropriate amines in 0.01N hydrochloric acid containing 1% (w/v) phenol. This was because it was planned to elute the amines from the phenol chromatogram in 0.01N hydrochloric acid and 1% was an estimate, which later investigations indicated to be rather high, of the amount of phenol liable to be present in such eluates. Only when a method had been shown to give a linear and reproducible relationship between fluorescence intensity and amine concentration in the presence of this amount of phenol, was it used to analyse chromatogram eluates.

All assays were carried out using a test-tube rack designed to protect the samples from light during fluorophor development. This rack consisted of a solid oblong piece of wood having several cylindrical holes bored into it, each hole sufficiently wide and deep to accommodate a 15 ml test-tube so that only the top 2 cm of the tube protruded from the hole. This precaution was taken in view of Sharman's report (Sharman, 1960, p. 29) that light lowered the intensity of the adrenaline and noradrenaline fluorophors.

#### Noradrenaline and Dopa

Two methods were used to assay these compounds, one being a modification of Häggendal's (1962) technique and the other a modification of a method of Bertler et al. (1958).

(1) Häggendal's method for the estimation of noradrenaline and dopa. 1.50 ml of a chromatogram eluate or of a standard solution of noradrenaline or dopa in 0.01N hydrochloric acid was adjusted to pH 6.5 by adding 1.0 ml of a mixture of equal volumes of 1M pH 6.5 sodium phosphate buffer and 0.6M pH 6.5 sodium citrate buffer. At zero time, 0.20 ml 0.02M iodine solution was added to reagent blanks and aqueous standards and 1.00 ml 0.04M iodine solution to chromatogram eluates. After 3 min., 1.00 ml 5% (w/v)

sodium sulphite containing 2% (w/v) disodium edetate was added, followed by 1.00 ml 5N sodium hydroxide 30 sec. later. 8½ min. after addition of the iodine, 1.00 ml 10N acetic acid was added. The 1.00 ml 0.04M iodine was found to give a persistent iodine colour to eluates from the phenol chromatogram in addition to precipitating traces of phenol. The triiodophenol precipitate did not affect the sensitivity of the assay and could be satisfactorily removed by centrifugation before reading the supernatant in the spectrophotofluorimeter.

Removal of phenol by warming the chromatogram eluates at 40°C in an atmosphere of nitrogen (Crawford and Outschoorn, 1951) or by extraction into ether, carbon tetrachloride, benzene and petroleum ether (Crawford, personal communication), resulted in amine loss.

Reagent blanks and mixed standards of 100 µg noradrenaline plus 100 µg dopa in 1.50 ml 0.01N hydrochloric acid, together with internal standards of 100 µg noradrenaline plus 100 µg dopa added to 1.50 ml of each chromatogram eluate, were processed in parallel with each batch of samples. Mixed standards were satisfactory since, under the conditions of the noradrenaline assay, 100 µg dopa contributed no fluorescence and under the conditions of the dopa assay 100 µg noradrenaline contributed no fluorescence.

1.50 ml of each chromatogram eluate was used as a tissue faded blank by adding the sodium sulphite-EDTA solution 10 min. after the sodium hydroxide.

The fluorescence intensities of the samples were measured, between 30 and 60 min. after production of the fluorophor, at the excitation and emission wavelengths for maximal fluorescence of the noradrenaline derivative [380/490 m $\mu$  on the Farrand SPF and 400/490 m $\mu$  on the Aminco-Bowman SPF]. After 60 min. the noradrenaline fluorophor slowly declined in intensity, while the reading for the faded blank slowly rose. Excitation and emission spectra were recorded for all samples to check that their excitation and fluorescence peaks corresponded with those from authentic noradrenaline.

After measuring the fluorescence due to the noradrenaline fluorophor, the samples were heated for 30 min. at 60°C and then left overnight at room temperature, before measuring the fluorescence intensities at 330/380 m $\mu$ , the wavelengths for maximum fluorescence of the dopa derivative. A primary filter, Corning no. 9863, was inserted between the excitation monochromator and the sample, before taking readings at 330/380 m $\mu$  on the Farrand SPF. This was necessary to prevent visible light, passed when the excitation

diffraction grating on this instrument was set to wavelengths of 400 mμ or less, from reaching the sample (Sharman, 1960, p. 83). The heat treatment resulted in the formation of the dopa fluorophor while simultaneously reducing the amount of noradrenaline fluorophor to levels which did not interfere with the dopa assay. Irradiation with U-V light (Carlsson and Waldeck, 1958) could be used to form the dopa fluorophor, but heating was just as effective. Leaving overnight was necessary, to allow the readings to stabilise and reduce 'between duplicate' variation. A linear relationship was obtained between the relative fluorescence measurements and concentration over the range of 50 mμg to 200 mμg for noradrenaline and for dopa. The intensities of fluorescence derived from noradrenaline and dopa and the size of the blanks were unaffected by the presence of phenol during development of the fluorescence.

(ii) Adaptation of the method of Bertler et al. (1958) for the assay of noradrenaline and dopa. 1.0 ml 0.1M pH 6.5 phosphate buffer and 0.10 ml 0.25% (w/v) zinc sulphate ( $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ) were added to 1.00 ml chromatogram eluate or 1.00 ml of a standard solution of noradrenaline or dopa in 0.01N hydrochloric acid. At zero time, there was added 0.10 ml 0.25% (w/v)



potassium ferricyanide; at 2 min., 0.10 ml freshly prepared 2% (w/v) ascorbic acid and at 2½ min., 0.9 ml 5N sodium hydroxide, with thorough mixing after each addition. Tissue blanks were prepared as follows: zero time, 0.10 ml 2% ascorbic acid; 2 min. 0.9 ml 5N sodium hydroxide and 2½ min., 0.10 ml 0.25% potassium ferricyanide. Faded blanks, analogous to those used in Häggendal's method (p. 50) in which the reducing agent (in this case, ascorbic acid) was added 10 min. after the sodium hydroxide, were abandoned because they gave anomalously high readings with 1.0 ml 0.01N hydrochloric acid containing 1% (w/v) phenol and 140 µg ascorbic acid. Ascorbic acid had the same R<sub>f</sub> as noradrenaline on chromatograms developed in phenol: hydrochloric acid. 140 µg ascorbic acid was the greatest amount that could be expected in a 1.0 ml aliquot of a 'noradrenaline-dopa' eluate from a chromatogram, assuming all the added ascorbic acid (p. 39) finished up in this eluate.

Each eluate was divided into four 1.0 ml aliquots for sample, tissue blank, internal standard of 100 µg noradrenaline and internal standard of 100 µg dopa respectively. Reagent blanks and separate aqueous standards of 100 µg amounts of noradrenaline and dopa were run in parallel with each batch of samples. The samples were centrifuged to get rid of any filter paper particles originating from the

chromatogram eluates. Within 30 min. of developing the fluorophors, the fluorescence intensities of the samples were read at 360/480 mμ and 400/520 mμ which are excitation/fluorescence maxima for the dopa and noradrenaline derivatives respectively. Both the Aminco-Bowman SPF and the Farrand SPF were used in these assays. The amounts of noradrenaline and dopa in a sample were calculated as follows:-

If:  $N_{360}$  = relative fluorescence intensity from  
100 mμg noradrenaline at 360/480 mμ

$N_{400}$  = relative fluorescence intensity from  
100 mμg noradrenaline at 400/520 mμ

$D_{360}$  = relative fluorescence intensity from  
100 mμg dopa at 360/480 mμ

$D_{400}$  = relative fluorescence intensity from  
100 mμg dopa at 400/520 mμ

$S_{360}$  = relative fluorescence intensity of the  
sample aliquot (after subtraction of the  
blank) at 360/480 mμ

$S_{400}$  = relative fluorescence intensity of the  
sample aliquot (after subtraction of the  
blank) at 400/520 mμ

Then:

$$n \frac{(N_{360})}{100} + d \frac{(D_{360})}{100} = S_{360}$$

$$n \frac{(N_{400})}{100} + d \frac{(D_{400})}{100} = S_{400}$$

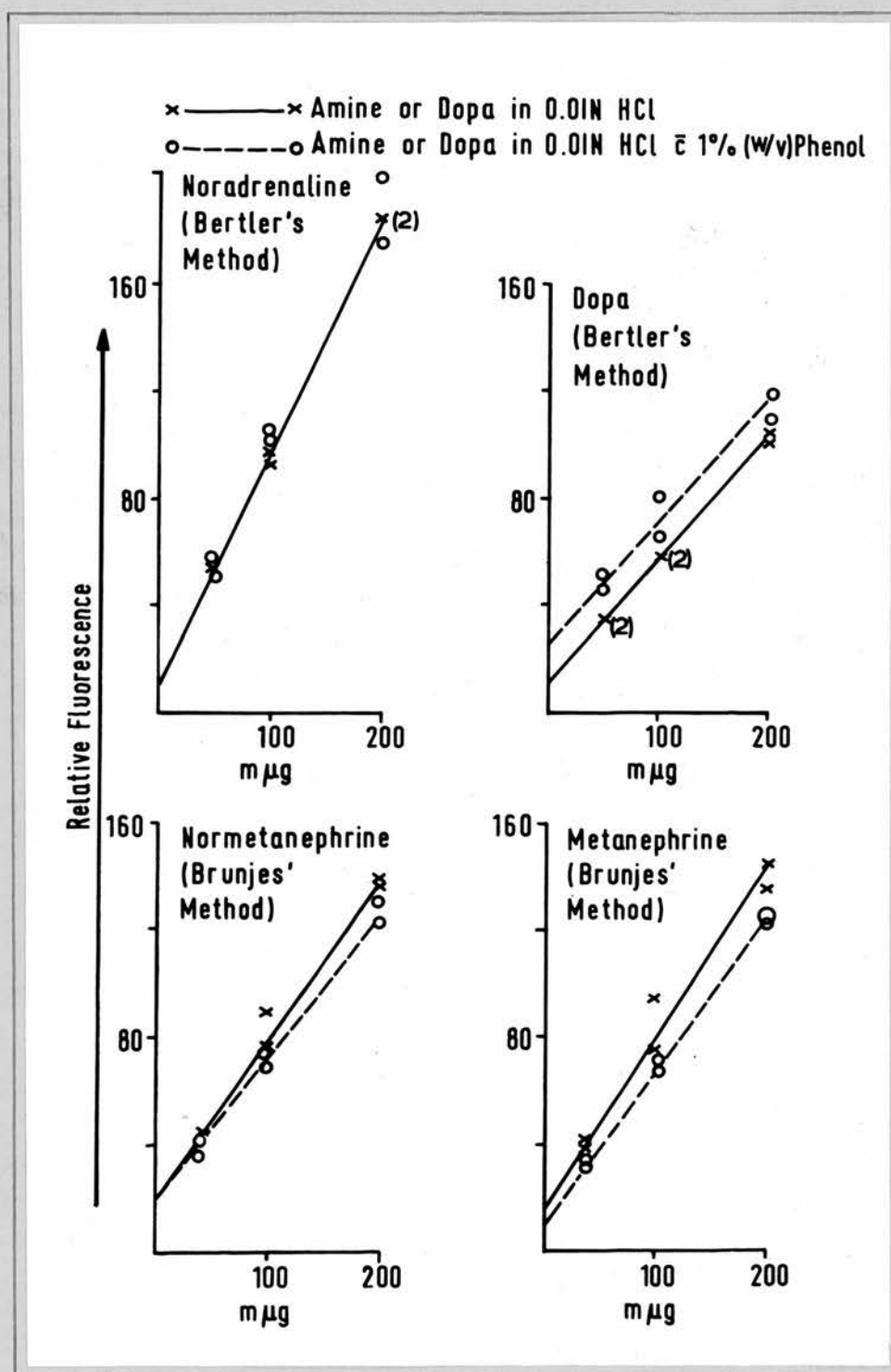


Solution of these equations gave the value for n (noradrenaline in  $m\mu g$  in sample aliquot) and for d (dopa in  $m\mu g$  in sample aliquot).

In this method phenol was not removed before fluorophor development and produced a fluorescence peak of its own at 330/460  $m\mu$ , the intensity of which was proportional to the amount of phenol present. This peak was presumably due to oxidation of phenol by potassium ferricyanide since it did not occur if the latter was omitted. A fluorescence peak at characteristic wavelengths was obtained from as little as 25  $m\mu g$  noradrenaline despite the presence of 1% (w/v) phenol, because the phenol peak at 330/460  $m\mu$ , was sufficiently distant from the noradrenaline fluorophor peak at 400/520  $m\mu$ . No fluorescence peak at the wavelength characteristic of the dopa fluorophor could be detected from amounts of dopa less than 200  $m\mu g$  since it was submerged in the phenol fluorescence spectrum because the dopa fluorophor had almost exactly the same fluorescence characteristics as the fluorescence derived from phenol. Nevertheless although 1% (w/v) phenol increased the blank in the dopa assay, thus reducing the sensitivity of the dopa assay, it did not increase the blank in the noradrenaline assay and did not affect the linearity or intensity of fluorescence

Figure 8

Calibration Curves for Noradrenaline, Dopa, Normetanephine,  
and Metanephine.



derivable from either noradrenaline or dopa (Figures 8 and 8 ). The following results were obtained on assaying two mixtures of noradrenaline (200 m $\mu$ g) and dopa (20 m $\mu$ g) in 1.0 ml 0.01N hydrochloric acid containing 1% (w/v) phenol and 140  $\mu$ g ascorbic acid:-

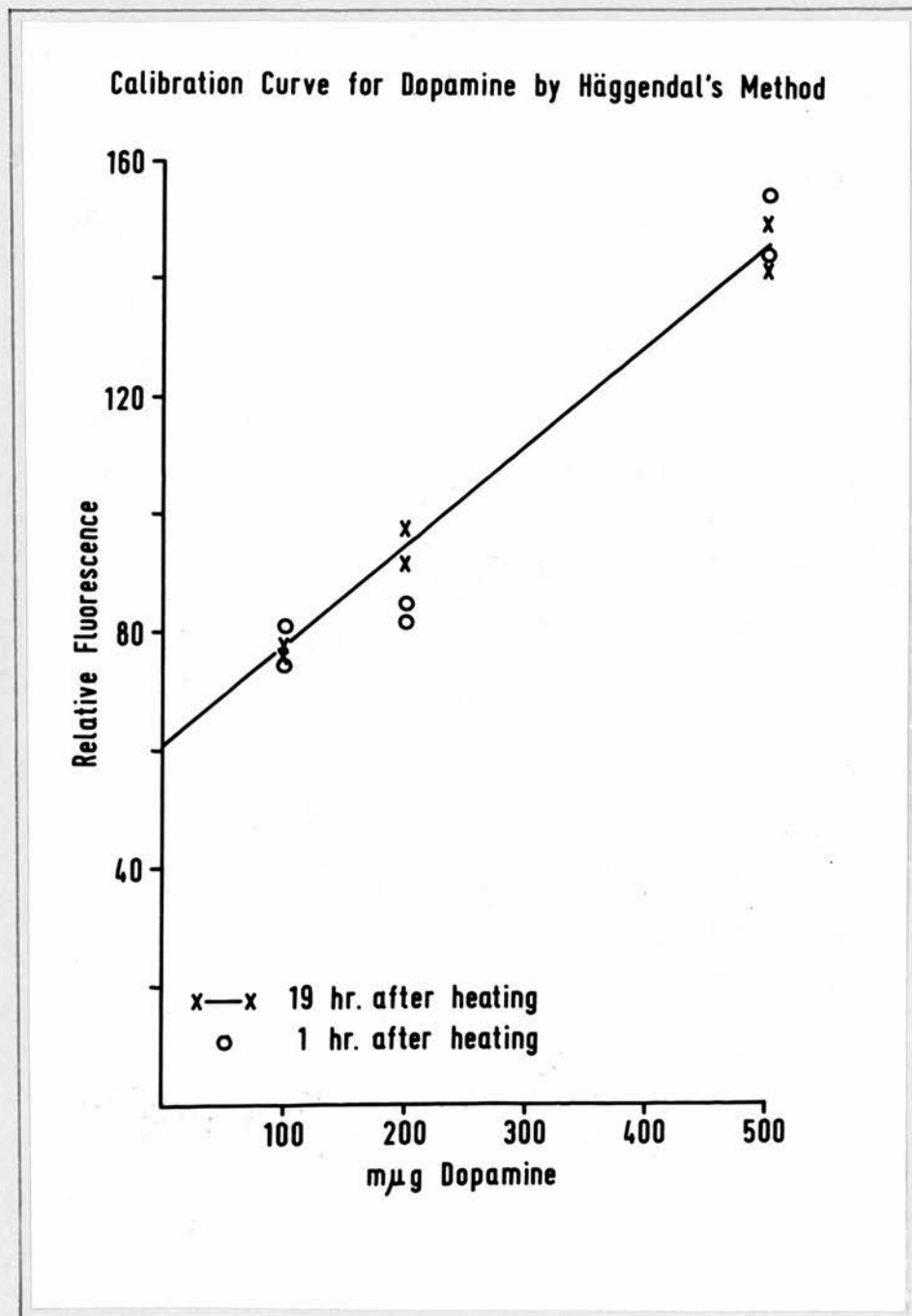
	Noradrenaline recovered	Dopa recovered
Mixture 1:	202 m $\mu$ g	19 m $\mu$ g
Mixture 2:	207 m $\mu$ g	20 m $\mu$ g

Estimation of mixtures containing less dopa was not attempted because <sup>20-</sup>30 m $\mu$ g was considered to be the limit of detection of dopa in a chromatogram eluate (Table 1 and p. 80 ).

Adrenaline, but not dopamine, normetanephrine or metanephrine, could also be estimated using this method of fluorophor production.

#### Adrenaline and Dopamine

Occasionally there was a sufficient separation of adrenaline from dopamine on the chromatogram for these two amines to be eluted and assayed separately, but more often they had to be eluted and assayed together. They were estimated in chromatogram eluates in exactly the same way as method (i) (p. 48 ) for noradrenaline and dopa. Mixed standards could

Figure 9

be used because 100  $\mu\text{g}$  adrenaline did not contribute to the dopamine assay nor 100  $\text{m}\mu\text{g}$  dopamine to the adrenaline assay. Peak adrenaline fluorescence was at 410/510  $\text{m}\mu$  on the Farrand SPF and 425/510  $\text{m}\mu$  on the Aminco-Bowman SPF. Peak dopamine fluorescence was at 330/380  $\text{m}\mu$  on both instruments. As in the dopa assay (p. 50 ), a primary filter, Corning no. 9863, was used when reading at 330/380  $\text{m}\mu$  on the Farrand SPF.

A linear relationship was obtained between the relative fluorescence measurements and concentration, from 50  $\mu\text{g}$  to 500  $\mu\text{g}$  for adrenaline and for dopamine (Figure 9 ). Figure 9 also demonstrates that leaving the samples for 19 hr. after heating stabilised, and increased the precision of, the dopamine readings, as it did the dopa readings (p. 51 ). The intensities of fluorescence derived from adrenaline and dopamine and the size of the blanks were unaffected by the presence of phenol during development of the fluorescence. Various unsuccessful attempts were made to improve upon the sensitivity, which was only 85  $\text{m}\mu\text{g}$ , of this dopamine assay. Oxidation with potassium ferricyanide (Carlsson and Waldeck, 1964) produced a derivative the fluorescence of which was less intense than that produced by iodine (p. 19 ).

### Metanephrine and Normetanephrine

These two amines were eluted separately from the chromatogram and assayed (i) using a modification of Brunjes' et al. (1964) method or (ii) using a modification of Häggendal's method, described on p. 48

(i) Modification of Brunjes' method for the assay of metanephrine and normetanephrine. 0.50 ml 0.5% (w/v) zinc sulphate ( $\text{Zn SO}_4 \cdot 7\text{H}_2\text{O}$ ) in 0.02N hydrochloric acid was added to 1.00 ml or 1.50 ml sample or standard metanephrine or normetanephrine in 0.01N hydrochloric acid. At zero time, 0.50 ml 0.075% (w/v) potassium ferricyanide (a freshly prepared dilution of a 0.25% (w/v) stock solution which was stored in a dark bottle in a refrigerator) was added and, at 4 min., 0.50 ml acetate buffer pH 5.6 (2.22 ml 10M acetic acid + 4 ml 5N sodium hydroxide to 100 ml) was added. At 8 min., 0.50 ml of a freshly prepared tautomerising solution made by mixing 1.00 ml freshly prepared 2% (w/v) ascorbic acid with 0.30 ml redistilled ethylenediamine and 8.7 ml 5N sodium hydroxide, was added.

The fluorescence from metanephrine and normetanephrine was reduced by 11% if the samples were exposed to light during the assay, instead of being protected from light by enclosing the tubes in the solid wooden test-tube rack (p. 48 ) used routinely for the assays.

Three 1.50 ml aliquots of the paper eluates were used for sample, internal standard and blank. Sample blanks were faded blanks, that is, at 8 min. only 0.44 ml 5N sodium hydroxide was added, and, after a further 10 min., 0.06 ml of a mixture of 1.00 ml 2% (w/v) ascorbic acid and 0.3 ml ethylenediamine was added. The samples were centrifuged to remove filter paper particles (p. 52 ), and the fluorescence read on the Aminco-Bowman SPF or the Farrand SPF between 20 to 40 min. after addition of the ferricyanide. On both instruments the fluorescence of the metanephrine fluorophor was maximal at 410/520 m $\mu$ . The fluorescence from normetanephrine was maximal at 400/505 m $\mu$  on the Aminco SPF and at 390/500 m $\mu$  on the Farrand SPF.

As in method (ii) for the assay of noradrenaline and dopa (p. 51 ) phenol was not removed by precipitation and gave rise to a fluorescence peak at 330/460 m $\mu$ . The peak from 1% (w/v) phenol completely masked that, at 400/505 m $\mu$ , from the fluorophor derived from amounts of normetanephrine of 200 m $\mu$ g and less. Since the metanephrine peak, at 410/520 m $\mu$ , was rather further away from the phenol peak, it was possible to obtain a fluorescence peak from as little as 100 m $\mu$ g metanephrine in the presence of 1% (w/v) phenol. The assay sensitivities for metanephrine and normetanephrine were reduced by about 10% by 1% (w/v) phenol.



Linear relationships were obtained between the relative fluorescence intensity and concentration for 40  $\mu$ g to 200  $\mu$ g amounts of the amines in the absence or presence of 1% (w/v) phenol (Figure 8 ).

Adrenaline and noradrenaline, but not dopamine, could be assayed by this method.

(11) Modification of Häggendal's method for the assay of metanephrine and normetanephrine. Häggendal's method (p. 48 ) could be used to assay metanephrine and normetanephrine in chromatogram eluates if bromine water was used to precipitate the phenol previous to iodine oxidation. No fluorophor was produced from metanephrine and normetanephrine if excess iodine was used to precipitate the phenol. Bromine water was added, drop by drop, with continuous tapping of the tube to ensure thorough mixing, until a faint persistent yellow colour was obtained. The yellow colour was discharged with the minimum amount of 5% (w/v) sodium sulphite containing 2% sodium edetate (w/v) added dropwise. The faintly yellow flocculent precipitate was removed by filtration through a small pledget of cotton-wool in the stem of a small filter funnel because it did not pack down well on centrifugation. An aliquot of the filtrate was then subjected to the assay procedure described on p. 48 . The disadvantages of this technique were its awkwardness and the reduction of

sensitivity by 50% due to retention of half the sample by the cotton-wool filter. Failure to remove the precipitate of tribromophenol prior to assay resulted in formation of a flocculent precipitate in the final stages of the assay and in no fluorophor formation. Taking into account the 50% loss caused by filtration, the relative fluorescence intensities of the fluorophors derived from metanephrine and normetanephrine were not diminished by the bromine water procedure and, in the case of normetanephrine the intensity was slightly increased. On the Farrand SPF, the fluorophor from metanephrine peaked at 410/510 m $\mu$  and the fluorophor from normetanephrine at 380/510 m $\mu$ .

TABLE 1

Sensitivities (in  $\mu\text{g}$ ) of Assay of Amines and Dopa in 0.01N HCl and Eluates from Brain Phenol Chromatograms (Paper Eluates) by different Methods of Assay.

Ranges in sensitivities given in parenthesis are from 4 estimations.

Sensitivity expressed as that amount of amine giving rise to the same fluorescence intensity as its appropriate blank.

Method of Assay	Noradrenaline		Dopa		Dopamine		Adrenaline		Normetanephrine		Metanephrine	
	0.01N HCl	Paper eluate	0.01N HCl	Paper eluate	0.01N HCl	Paper eluate	0.01N HCl	Paper eluate	0.01N HCl	Paper eluate	0.01N HCl	Paper eluate
Häggendal	15 (10-20)	30 (10-40)	80 (80-100)	80 (60-100)	85 (50-100)	85 (50-100)	15 (10-20)	30 (20-40)	50	200	60	200
Bertler	20 (20-25)	20 (10-30)	40 (30-50)	60 (50-70)								
Brunjes									20 (10-20)	35 (30-50)	10 (5-15)	20 (15-20)

TABLE 2

Lowest detectable concentrations of amines and dopa in rat brain ( $\mu\text{g/g}$ ). Calculated assuming the limit of detection equalled half the sensitivity.

Method of assay	Nor-adrenaline	Dopa	Dopamine	Adrenaline	Nor-metanephrine	Meta-nephrine
Häggendal	0.11	0.22	0.30	0.11	0.58	0.58
Bertler	0.12	0.16				
Brunjes					0.12	0.06

## RESULTS

The sensitivities of assay of the amines and dopa both in 0.01N hydrochloric acid solution and in eluates from chromatograms of extracts of rat brain, by all the methods which have been described, are given in Table 1 . The sensitivities of assay in chromatogram eluates did not differ greatly from that in pure solution because, for the most part, the blanks from the eluates were about the same as the reagent blanks and quenching of fluorescence (as indicated by %  $\frac{\text{Internal standard}}{\text{Aqueous standard}}$  in Table 3 ) by the eluates was not marked.

It was considered that the least amount of amine and dopa detectable in any assay was that amount giving rise to a fluorescence intensity equal to half its appropriate blank. The lowest concentration detectable of each amine and dopa in whole rat brain was calculated on this basis (Table 2 ).

Recovery of Amines and Dopa through the  
Chromatographic Procedure

A mixture containing 500  $\mu\text{g}$  of noradrenaline, dopamine and normetanephrine and 300  $\mu\text{g}$  of dopa, adrenaline and metanephrine was applied in 0.15 ml 80% (v/v) methanol containing 25 mg % (w/v) ascorbic

TABLE 3

Recovery of Noradrenaline, Dopamine and Normetanephine (500 mug each) and Dopa, Adrenaline and Metanephine (300 mug each) from a mixture in pure solution, taken through Chromatography in NaCl:HCl followed by phenol:HCl.

Experiment carried out in duplicate

Int. Std. = fluorescence from 100 mug amine or dopa added to portion of chromatogram eluate  
fluorescence from 100 mug amine or dopa in pure solution

Exp.	Noradrenaline			Dopa			Dopamine			Adrenaline			Metanephine			Normetanephine		
	Procedure recovery %	Int. Std. Aq. Std. %	Procedure recovery %	Int. Std. Aq. Std. %	Procedure recovery %	Int. Std. Aq. Std. %	Procedure recovery %	Int. Std. Aq. Std. %	Procedure recovery %	Int. Std. Aq. Std. %	Procedure recovery %	Int. Std. Aq. Std. %	Procedure recovery %	Int. Std. Aq. Std. %	Procedure recovery %	Int. Std. Aq. Std. %	Procedure recovery %	Int. Std. Aq. Std. %
1	92		77		41		55		119		56		100		100		100	
2	82	93	72	lost	61	83	45		95		85							
		78		92		82		60				100						100



TABLE 4

Amines and Dopa ( $\mu\text{g/g}$ ) in Rat Brain  
Estimates uncorrected for procedure recoveries

$\frac{\text{Int. Std.}}{\text{Aq. Std.}} = \frac{\text{fluorescence from 100 } \mu\text{g amine or dopa added to portion of chromatogram eluate}}{\text{fluorescence from 100 } \mu\text{g amine or dopa in pure solution}}$

Assay of noradrenaline and dopa: H indicates assay-Haggendal's method (p.48); B by Bertler's method (p.51)

Assay of adrenaline and dopamine: by Haggendal's method (p.56)

Assay of metanephrine and normetanephrine: by Brunjes' method (p.59)

Experiment	Samples	Noradrenaline			Dopa			Dopamine			Adrenaline			Normetanephrine			Metanephrine			
		μg/g	Procedure recovery %	Int. Std. Aq. Std. %	μg/g	Procedure recovery %	Int. Std. Aq. Std. %	μg/g	Procedure recovery %	Int. Std. Aq. Std. %	μg/g	Procedure recovery %	Int. Std. Aq. Std. %	μg/g	Procedure recovery %	Int. Std. Aq. Std. %	μg/g	Procedure recovery %	Int. Std. Aq. Std. %	
(1)	Duplicate samples	1	0.26 <sup>H</sup>		48	3.0 <sup>H</sup>		78	2.65		92	<0.11		57						
		2	0.30 <sup>H</sup>		35	3.48 <sup>H</sup>		83	2.03		106	<0.11		36						
	Duplicate recoveries	3		87 <sup>H</sup>	44		900 <sup>H</sup>	56		156	66		74	54	Not done			Not done		
		4		67 <sup>H</sup>	49		1320 <sup>H</sup>	28		59	87		47	54						
(2)	Duplicate samples	1	0.26 <sup>H</sup>		70	5.9 <sup>H</sup>		lost						<0.12		65	<0.06		70	
		2	0.36 <sup>B</sup>		95	0.12 <sup>B</sup>		72	Not done			Not done			0.09		80	<0.06		64
	Duplicate recoveries	3		68 <sup>H</sup>	84		231 <sup>H</sup>	80								72	85		48	80
		4		lost			lost									72	72		36	76
(3)	Duplicate samples	1	0.30 <sup>B</sup>		70	0 <sup>B</sup>		75	1.11		104	<0.11		80	0.19		64	<0.06		79
		2	0.28 <sup>B</sup>		104	0 <sup>B</sup>		78	0.87		112	<0.11		80	0.14		47	<0.06		76
	Duplicate recoveries	3		40 <sup>B</sup>	82		125 <sup>B</sup>	67		73	124		93	80		33	95		57	69
		4		42 <sup>B</sup>	100		98 <sup>B</sup>	84		42	114		46	80		99	31		33	70



acid, to a chromatogram which was developed as described on p. 39. Normetanephrine and metanephrine were assayed by Brunjes' method and the other amines and dopa by Häggendal's method. The experiment was carried out in duplicate. The recoveries of each amine are given in Table 3. The eluted amines showed the same fluorescence characteristics as their respective standards.

#### Estimation of Amines and Dopa in Rat Brain

The results of three experiments are given in Table 4. To determine the recoveries of the amines and dopa through the procedure, 750  $\mu\text{g}$  each of noradrenaline, dopamine and normetanephrine and 300  $\mu\text{g}$  each of adrenaline, dopa and metanephrine were added to two portions of rat brain homogenate in each experiment, as described on p. 36. Additions of metanephrine and normetanephrine and of adrenaline and dopamine were omitted in experiments (1) and (2), respectively, in order to limit the number of samples for estimation. The concentrations of the amines and of dopa are expressed as  $\mu\text{g/g}$  wet tissue and calculated on the basis of the appropriate internal standards. They are uncorrected for procedure recoveries. Some noradrenaline and dopa samples were estimated by Häggendal's method (p. 48) and some by Bertler's method

(p. 51 ). Metanephrine and normetanephrine were assayed by Brunjes' method (p. 59 ), which was used in preference to Häggendal's method because it was simpler and more sensitive (Table 1 ).

#### Noradrenaline and Dopa

The 'noradrenaline' brain samples and procedure recoveries assayed by Häggendal's or Bertler's method showed maximal fluorescence at the same wavelengths as the noradrenaline aqueous and internal standards. These wavelengths were different from the wavelengths of maximal fluorescence of the blanks which were 410/500 mμ for the sample faded blank in Häggendal's method on the Farrand SPF and 400-420/460 mμ for the sample faded blank in Bertler's method on the Aminco-Bowman SPF. The values for brain noradrenaline whether assayed by Häggendal's or Bertler's methods were reasonably consistent, despite the fact that the procedure recoveries ranged from 40 to 89%. Since dopa has never been detected in normal rat brain (Bertler, Falck and Rosengren, 1963; Anton and Sayre, 1964), its concentration in this tissue must be zero or very low; Anton and Sayre (1964) quote a concentration of less than 0.05 μg/g. Häggendal's method, however, gave values for rat brain dopa of 3.0, 3.48 and 5.9 μg/g in three separate samples (Table 4 ). These high figures

were attributed to a substance which fluoresced, like dopa, at 330/380 mμ. This substance was not ascorbic acid, which was known to run to about the same place as noradrenaline on the phenol chromatogram and could therefore be present in the noradrenaline-dopa eluates. Since this high fluorescence was not obtained from chromatograms of pure solutions (Table 3), it must originate from the brain itself. An alternative 'unprecipitated blank', in which phenol was not precipitated, was tried, in the hope that it might include this interfering substance. 5% (w/v) sodium sulphite containing 2% (w/v) sodium edetate (p. 49) was added first, followed by iodine and the other reagents in their usual order. However, this blank gave the same reading as the faded blank and peaked, like both faded and reagent blanks, at 330/380 mμ. The interfering substance did not appear to fluoresce when Bertler's method was used to estimate noradrenaline and dopa. Much more reasonable figures were obtained for rat brain dopa using this method, and the procedure recoveries were satisfactory. Ascorbic acid, if present in these eluates, was very much less than the estimated 140 μg (p. 52).

The fluorescence peak due to the phenol in a 1.5 ml aliquot of a chromatogram eluate was very much less than that produced from 1.5 ml 0.01N hydrochloric

acid containing 1% (w/v) phenol, inferring that 1% was an over-estimate of the amount of phenol present in the chromatogram eluates. If, as in experiment (3), the developed chromatograms were washed with four instead of two portions of benzene, this peak was very small. Peak fluorescence from the procedure recoveries, since they contained 2.5 times as much noradrenaline as dopa, occurred at the wavelengths of maximum fluorescence from noradrenaline (i.e. 400/520 mμ). These samples gave, at most, only slight indication of a peak with the wavelength characteristics (360/480 mμ) of dopa.

#### Adrenaline and Dopamine

No adrenaline could be detected in rat brain. The fluorescence from the procedure recoveries of adrenaline, which varied from 46% to 74%, like that from the internal adrenaline standards, was maximal at 425/510 mμ. On the Farrand SPF, the sample faded blank gave a small excitation peak at 410 mμ, i.e. at the wavelength for maximum excitation of the adrenaline fluorophor but it showed no fluorescence peak at 510 mμ, when excited at 410 mμ. The adrenaline fluorophor could thus be distinguished from the blank by the fact that, unlike the blank, it showed a fluorescence peak at 510 mμ. The dopamine fluorophor, however, could not be distinguished from its blank because fluorophor

TABLE 5

Dopamine (µg) in eluates from dopamine and adrenaline positions of chromatograms of rat brain extracts

Experiment	Sample		Dopamine in dopamine position	Dopamine in adrenaline position	Total dopamine
(1)	Duplicate	1	1510	233	1743
	Samples	2	1470	262	1732
	Duplicate	3	1530	1040	1570
	Recoveries	4	1080	550	1630
(2)	Duplicate	1	151	497	648
	Samples	2	228	286	514
	Duplicate	3	90	802	892
	Recoveries	4	197	553	750

and blank had both excitation and fluorescence maxima at the same wavelengths viz. 330 mμ and 380 mμ, respectively. The values for rat brain dopamine in experiments (1) and (3) are respectively about 3 times and 1.5 times those quoted in the literature (Bertler and Rosengren, 1959; Anton and Sayre, 1964) inferring that there might be present in the dopamine eluates a material originating from brain which was similar, though less in amount, to that found in the dopa eluates. The 'unprecipitated' blank from the dopamine eluates was the same as the faded blank, suggesting that any interfering substance in the dopamine eluates behaved similarly, under the conditions of blank formation, to that present in the dopa eluates. The procedure recoveries for dopamine varied from 42% to 156%.

In <sup>some</sup> experiments ~~(1) and (3)~~ adrenaline and dopamine were eluted separately. Despite apparently adequate separation on the marker chromatograms, some dopamine or dopamine-like substance was found in eluates from the adrenaline position of all chromatograms of brain extracts (Table 5) and in two out of four procedure recoveries adrenaline was recovered from the dopamine position instead of the adrenaline position.

#### Metanephrine and Normetanephrine

The values obtained for rat brain normetanephrine were rather suspect because the normetanephrine samples,

unlike the 'recovery' samples, did not give a fluorescence peak at 400/500 mμ, the wavelength of maximal fluorescence of the normetanephrine fluorophor. Both samples and blanks produced a fluorescence peak at 330/420 mμ, which was due to phenol leached from the chromatogram. This peak was considerably less than that produced by 1% (w/v) phenol, corroborating the evidence obtained from assays of noradrenaline-containing eluates by Bertler's method (p. 7/ ), that the amount of phenol present in the chromatogram eluates was less than 1%. As little as 100 mμg normetanephrine could be detected, as a fluorescence peak at 400/500 mμ, in these phenol-containing eluates. A major disadvantage of this technique for assaying normetanephrine was the rather poor chromatographic separation of normetanephrine from adrenaline (Figure 7 ). Any adrenaline present in the normetanephrine eluate would be assayed as normetanephrine (p. 6/ ) and thus give rise to falsely high normetanephrine values.

No metanephrine could be detected in rat brain. The fluorescence peaks obtained from samples and blanks were the same and had the wavelength characteristics (330/420 mμ) of the phenol fluorophor. 20 mμg to 50 mμg metanephrine should give rise to a fluorescence peak at 410/520 mμ in the presence of chromatogram



eluates containing phenol. Metanephrine was well separated on the chromatogram from all other amines.

Effect of Different Methods of Extraction  
on the Estimation of Amines and Dopa in Rat Brain

Several methods of extraction, different from that described on p. 36 were investigated. Method (i) was used by Ashcroft, Eccleston and Crawford (1965) for the estimation of the 5-hydroxyindoles in rat brain. It was originally hoped that, by pooling a sufficient number of rat brains, it would be possible to use part of such an extract for the estimation of the 5-hydroxyindoles and part for the estimation of the catechol derivatives. Such a technique would allow indole-alkylamine and catecholamine metabolic pathways to be followed simultaneously from the same brains. Method (ii) was an attempt to remove the substance interfering in the dopa and dopamine assays (p. 73 ) by extraction of this substance into isoamyl alcohol or ethyl acetate.

(i) Protein Precipitation with Acetic Acid and Acetone  
compared with Perchloric Acid

Rat brains were homogenised in acetic acid, protein precipitated with acetone and lipids removed by extraction into petroleum ether, as described by

Ashcroft et al. (1965). The ensuing extract, reported to give a good separation of 5-hydroxyindoles on paper chromatography using butanol:acetic acid as the developing solvent (Ashcroft et al., 1965), when applied to a chromatogram was found to form a yellow greasy layer impermeable to the aqueous hydrochloric acid:sodium chloride solvent. The material responsible for this deposit could be removed by adding sufficient 4N perchloric acid to the extract, after it had been shaken with petroleum ether, to produce a 0.1N perchloric acid extract solution. On processing this solution as on p. 36, an extract was obtained which was easily applied to chromatography paper and did not affect the Rfs of marker amounts of the amines and dopa in hydrochloric acid:sodium chloride and phenol:hydrochloric acid. Recoveries of adrenaline, noradrenaline, dopa and dopamine added to rat brain and taken through this procedure were very poor and the brain noradrenaline values very low, probably due to the lengthiness of the procedure. This extraction method was therefore rejected in favour of the shorter method of homogenising the brain directly in perchloric acid (p. 36), which gave better recoveries and values for noradrenaline in rat brain similar to those reported in the literature (Pletscher, Bartholini, Bruderer, Burkard and Gey, 1964; Anton and Sayre, 1964).

(11) Effect of Extraction of Perchloric Acid Extracts  
with Isoamyl Alcohol and Ethyl Acetate

In one experiment ((4) in Table 6 ), the perchloric acid supernatants from the brain homogenate were shaken with 10 volumes washed isoamyl alcohol for 3 min., centrifuged, and the alcohol removed by suction. The pH of the aqueous phase was then adjusted to 4 and the samples processed as on p. 37. Isoamyl alcohol was used because Axelrod, Inscoe, Senoh and Witkop (1958) found it removed substances interfering in the estimation of urinary metanephrine and normetanephrine.

In a second experiment ((5) in Table 6 ) the perchloric acid supernatants were saturated with sodium chloride and extracted 3 times with an equal volume of ethyl acetate before adjusting the pH to 4 and processing as on p. 37. The effect of extraction with ethyl acetate was investigated because in the future it was hoped to separate the acid metabolites of the amines from the amines by extraction of the acids into ethyl acetate.

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Footnote: Washed isoamyl alcohol : 500 ml isoamyl alcohol was washed with 100 ml 1N sodium hydroxide, 100 ml 1N hydrochloric acid and with 2 x 100 ml distilled water. The water washes were removed after centrifugation.

TABLE 6

Effect of extraction with isoamyl alcohol and ethyl acetate on amines and dopa ( $\mu\text{g/g}$ ) in rat brain.

Estimates uncorrected for procedure recoveries

Int. Std. = fluorescence from 100  $\mu\text{g}$  amine or dopa added to portion of chromatogram eluate  
Aq. Std. = fluorescence from 100  $\mu\text{g}$  amine or dopa in pure solution

Amines and dopa all measured by Haggendal's method (p.48)

Experiment	Samples	Noradrenaline			Dopa			Dopamine			Adrenaline		
		$\mu\text{g/g}$	Procedure recovery %	Int. Std. Aq. Std. %	$\mu\text{g/g}$	Procedure recovery %	Int. Std. Aq. Std. %	$\mu\text{g/g}$	Procedure recovery %	Int. Std. Aq. Std. %	$\mu\text{g/g}$	Procedure recovery %	Int. Std. Aq. Std. %
(4) Extracted with isoamyl alcohol	Duplicate Samples	1 0.06		71	0.42		115	0.81		122	<0.11		57
		2 0.20		61	1.24		124	1.66		100	<0.11		56
	Duplicate Recoveries	3	Not done			290	132		94	73		Not done	
		4	Not done			80	135		93	73		Not done	
(5) Extracted with ethyl acetate	Duplicate Samples	1 0.08		100	<0.22		122	1.51		97	<0.11		76
		2 0.09		86	<0.22		67	0.75		105	<0.11		70
	Duplicate Recoveries	3	16	87		87	130		0	118		0	69
		4	lost	lost		lost	lost		0	55		0	61

The results of these two experiments are given in Table 6 . Comparison of the results in Table with those in Table 4 (p. 67 ) shows that extraction with isoamyl alcohol lowered the dopa figures while extraction with ethyl acetate abolished them altogether. The material responsible for these high dopa estimates was presumably partially extracted into isoamyl alcohol and wholly extracted into ethyl acetate. Neither solvent extraction had any marked effect on the dopamine estimates but both considerably lowered the noradrenaline estimates.

## DISCUSSION

The relative fluorescence obtained from a constant amount of amine or dopa varied from run to run, possibly due to fluctuations in the light intensity of the xenon lamp and to variation in the intensity of light incident upon the samples during fluorophor development. Despite taking the precautions listed on p.28, the blanks obtained on different occasions from all assays, whether phenol was present or not, could vary by about  $\pm 50\%$  of the mean value. Sometimes, on subsequent investigation, the blame for an increased blank could be traced to a particular reagent. Since the sensitivity of an assay depended on the relative fluorescence obtained from a constant amount of the substance and from the blank (p.31) and since these two factors varied independently, a marked variation in sensitivities was obtained (Table 1).

The limits of detection of the amines and dopa in rat brain (Table 2, p. 64) are not as low as might be expected from the sensitivities of assay of each compound (Table 1, p. 63), because the actual amount quantitated in the SPF was only a fraction (about one fifth) of the total present in the original sample of tissue taken for analysis. This was because aliquots

were taken after homogenisation (p.36 ) and again after elution (p.48 ) when the eluate was divided into three portions for the estimation of sample, faded blank and internal standard.

The recoveries of two mixtures of the amines plus dopa in pure solution, taken through the chromatography and assay procedures (Table 3 , p.66 ) were, with the exception of adrenaline and possibly dopamine, satisfactory, showing that reasonably consistent results can be obtained using this technique. Adrenaline, being the least stable, is the most likely to be lost during processing. The recovery of noradrenaline was 10% and the recoveries of the other amines and dopa were 0% when, starting from pure solution, they were put through the tissue extraction procedure before chromatography. These poor recoveries were almost certainly due to the difficulty of adjusting the pH of unbuffered solutions. The recoveries were all much improved in the presence of brain tissue, extracts of which contain substances which can act as buffers and thus make more easy the adjustment of the pH. The reason for the variation in recoveries of noradrenaline, adrenaline and dopamine from tissue extracts (Table 4 , p.67 ) was not investigated at this stage but will be discussed in Section II of this thesis. Since these recoveries were much better through the chromatography alone, the variable



loss is most probably occurring at the extraction stage. In calculating the recoveries it was assumed that the added amines and dopa behaved like the endogenous amines and dopa in that they were evenly distributed between the supernatant and the water content of the tissue precipitate, estimated by Bertler et al. (1958) at 75% (v/w) of the tissue weight.

Acid acetone, like acid ethanol (Bertler et al., 1958) and unlike perchloric acid, was found to extract a large amount of lipid. Vogt (1959) reported that acid ethanolic brain extracts, which presumably contained lipid, did not affect the Rfs of the catecholamines when chromatographed in the phenol:hydrochloric acid solvent but this lipid was found (p. 76) to prevent the flow of an aqueous chromatographic solvent. Using the procedure of extraction with perchloric acid described on p. 36 extracts were obtained which were easily applied to the chromatogram and produced only a faint yellowish deposit on the paper. The degree of clarity of the supernatant obtained from the acid homogenate and the amount of the deposit visible at the origin of the chromatogram after application of the extracts varied slightly from experiment to experiment but there appeared to be no correlation between these factors and the final amine levels.

TABLE 7

Noradrenaline, normetanephrine, adrenaline and dopamine in rat brain ( $\mu\text{g/g}$ )  
by different workers using fluorimetric methods of assay

The three low noradrenaline estimates are averaged separately as well as together with the other noradrenaline estimates.

	Noradrenaline	Normetanephrine	Adrenaline	Dopamine
Montagu (1956)	0.44		0.21	
Montagu (1957)	0.22		0.07	0.64
Montagu (1963)	0.19		0.05	0.53
Anton and Sayre (1962)	0.15		0.04	
Murphy and Sourkes (1959)	0.50			0.89
Bertler et al. (1959)	0.49			0.60
Crout et al. (1961)	0.58			
Spector et al. (1962)	0.47			
Gunne (1963)	0.56		0.04	
Pletscher et al. (1964)	0.33			0.61
Anton and Sayre (1964)	0.32			0.68
Murphy and Sourkes (1961)				0.63

Carlsson and Lindquist (1962)		0.03		
Means	$\underbrace{0.20 \quad 0.46}_{0.38}$	0.03	0.05	0.65

Except in experiment (1) (Table 4 , p. 67 ), quenching of fluorescence by paper eluates was found not to be marked, but it was variable. This variability made difficult the comparison of the degree of quenching of fluorescence by eluates obtained from pure solution chromatograms with that caused by eluates obtained from brain chromatograms. It did, however, appear that, in general, the eluates from chromatograms of brain tissue produced no more quenching of catecholamine, methoxyamine or dopa fluorescence than did those from pure solution chromatograms.

The estimates for rat brain noradrenaline obtained in these experiments varied from 0.26 to 0.36  $\mu\text{g/g}$  (Table 4 , p. 67 ) with a mean of 0.29  $\mu\text{g/g}$ . The values for rat brain reported in the literature vary from 0.15 to 0.58  $\mu\text{g/g}$  with a mean of 0.38  $\mu\text{g/g}$  (Table 7 ). Significantly lower noradrenaline estimates were obtained if the acid extracts of brain were extracted with isoamyl alcohol or ethyl acetate (Table 6 , p. 78 ). It would appear therefore that there was some loss of noradrenaline into these solvents. This will be discussed in more detail in the second part of this section, in which an ethyl acetate extraction was incorporated into the general technique in order to separate the carboxylic acid metabolic derivatives of the catecholamines from those still

retaining an amino group. The procedure recoveries for noradrenaline varied from 40% to 87%. The sample estimates did not show such a wide variation, suggesting that the recovery of noradrenaline added to the perchloric acid homogenates might not accurately represent the recovery of the endogenous noradrenaline extracted from the brain. This important point will be discussed more fully in Section I, Sub-section 2 and in Section II.

Dopa has never been detected in normal, untreated brains (Anton and Sayre, 1964) and provided Bertler's method was used none was found in these experiments. Since maximum fluorescence from noradrenaline occurred at wavelengths close to those of maximum fluorescence from dopa (p. 53 ), discrete fluorescence peaks from these two amines could only be obtained when the concentration of each was about the same. When, as in the procedure recoveries (p. 68 ) the concentration of one was as much as  $2\frac{1}{2}$  times the concentration of the other, only one peak, at the wavelengths of maximum fluorescence of that compound present in the higher concentration, was demonstrable. In experiments in which a dopa estimate would be required, as for instance after dopa administration to the animals when the concentration of dopa might be considerably in excess of that of noradrenaline, only a dopa peak would be detectable.

A substance, present in the dopa and possibly also in the dopamine, eluates from brain chromatograms, reacted similarly to dopa in Häggendal's but not in Bertler's, method of estimation. It would appear therefore that the method of Bertler is the one of choice for the assay of dopa. No other information on the nature of this interfering substance is available apart from the fact that it appears to be extractable into polar solvents.

Rat brain has been reported to contain about 0.05  $\mu\text{g/g}$  adrenaline (Table 7, p. 83). This amount was too small to be detected by the method of assay reported in this section.

Rat brain dopamine estimates by other workers vary from 0.53 to 0.89  $\mu\text{g/g}$ , with a mean of 0.65  $\mu\text{g/g}$  (Table 7, p. 83). The dopamine estimates obtained using Häggendal's method were all equal to or greater than the upper limit of this range, possibly due to contamination with some of the 'interfering substance' already referred to. Dopamine was found in both 'dopamine' and 'adrenaline' eluates from two brain phenol chromatograms but only in the 'dopamine' eluate of a pure solution chromatogram. There was no indication that the dopamine estimate in either the 'dopamine' or the 'adrenaline' eluates was due entirely to the 'interfering substance'. A possible explanation for

the presence of dopamine in the adrenaline eluates is that extraction of dopamine with perchloric acid by the procedure on p. 36 gave rise to two forms of dopamine with different Rf values, analogous to the double spots found by Shepherd and West (1952) on chromatographing trichloroacetic acid solutions of adrenaline and dopamine, in butanol:acetic acid:water. This hypothesis was reconsidered on finding (p. 146) that the spread of acetyl-dopamine along a chromatogram was apparently greater than that indicated by its visualised marker. Assuming dopamine behaves similarly, it is possible that the 'adrenaline' chromatogram eluates would contain some dopamine which had spread from the immediately adjacent 'dopamine' position even though, judging by the markers, there was an apparent clear-cut separation of adrenaline from dopamine. The amount of dopamine on the pure solution chromatogram (500  $\mu$ g) was less than the amounts of dopamine on the brain extract chromatograms (Table 4, p. 67) and, because of the greater distance travelled by the solvent, the separation of adrenaline from dopamine was much better on the pure solution than on the brain extract, chromatograms. These facts could explain why no dopamine was found in the adrenaline eluates from the pure solution chromatograms.



The figures obtained for rat brain normetanephrine are considerably higher than that of  $0.03 \mu\text{g/g}$  reported by Carlsson and Lindquist (1962). It is unlikely that adrenaline, which was not well separated on the chromatogram from normetanephrine, would contribute to these high figures, because there is so little adrenaline present in rat brain (Table 7). Three out of the four normetanephrine recoveries were, unlike the recoveries for noradrenaline, adrenaline and dopamine, both satisfactory and consistent. This may be a reflection of the greater stability of normetanephrine compared with the catecholamines.

No metanephrine was detected in rat brain although Milhaud and Glowinski (1963) found radioactively labelled metanephrine in rat brain after intra-ventricular injection of  $3 \mu\text{g } C^{14}$ -dopamine, suggesting that metanephrine can be synthesised by the brain. No estimates of brain metanephrine could be found in the literature. The low metanephrine recoveries may have been due in part to inaccurate localisation on the chromatogram strip. This is possible because the positions of 'marker' metanephrine and normetanephrine were not always very distinctly visualised. In cases of doubt, normetanephrine could be safely assumed to lie just above the adrenaline position, but there was no such additional check on the position of metanephrine.

Attempts to improve the visualisation of metanephrine and normetanephrine by using iodine, which is a stronger oxidising agent than ferricyanide, to oxidise the amines before treating them with alkali, produced no increase in the intensity of the blue fluorescence seen under U-V light (p. 45). The choice of reagents to visualise catecholamines on phenol impregnated chromatograms is limited since reagents capable of detecting phenolic groups are valueless.

Methoxydopamine, the o-methylated metabolite from dopamine, is present in brain tissue (Carlsson and Waldeck, 1964). At the time of these experiments, there was no reported method for estimating methoxydopamine. Given such a method, estimation of methoxydopamine in the same sample as the other amines would be possible because it was satisfactorily separated from its nearest neighbours, metanephrine and normetanephrine, by chromatography in phenol:hydrochloric acid.

On reviewing the results obtained for the estimates of catecholamines, methoxyamines and dopa in rat brain, it became obvious that the methodology for their estimation, as described in this section, had severe limitations. The principal disadvantages were:

- (1) The presence of phenol in the chromatogram eluates complicated the assays. In those assays in which phenol

was not removed by precipitation, the fluorescence peak derived from the phenol tended to mask that derived from the amine.

(2) There was insufficient separation of the amines, so that adrenaline had to be measured in the presence of dopamine and noradrenaline in the presence of dopa. Although methods were developed which would assay separately the components of these mixtures, such methods are never as satisfactory as methods for assaying the individual separated compounds. In addition, normetanephrine was not very well separated from adrenaline. These two substances, unlike noradrenaline and dopa, cannot be assayed in the same eluate sample. Differential assay could be obtained by measuring the total amine in one sample and determining the catecholamine, e.g. by the ethylenediamine method (Weil-Walherbe and Bone, 1954), in another sample; a procedure which results in an undesirable increase in the least amount of each amine which can be detected.

(3) The sensitivity of dopamine assay was so poor that the amount of dopamine in whole rat brain as reported in the literature, was not much greater than the limit of sensitivity of the method. The figures for rat brain dopamine were suspiciously high, suggesting they included some other substance.

These disadvantages stem largely from the use of phenol as the chromatographic solvent. A method of separating the amines as their acetylated derivatives, using a phenol-free solvent system (Goldstein, Friedhoff, Simmons and Prochoroff, 1959<sup>b</sup>) was therefore investigated. This system had the additional advantage over phenol: hydrochloric acid that adrenaline, noradrenaline and dopamine were separated from one another and from their corresponding methoxy derivatives. Application of this method to the estimation of brain catechol- and methoxyamines is described in the next sub-section.

## SUB-SECTION 2

Separation of Adrenaline, Noradrenaline, Dopamine,  
Metanephrine, Normetanephrine, Methoxydopamine,  
and Dopa as their Acetylated Derivatives  
and their subsequent Estimation in  
Extracts of Rat Brain.

## METHODS

The possibility of separating the catecholamines and their methoxy derivatives from one another in the form of their acetyl derivatives as a preliminary to their estimation was suggested by the work of Goldstein, Friedhoff and Simmons (1959a) and Goldstein, Friedhoff, Simmons and Prochoroff (1959b). These workers separated the acetylated derivatives of the amines by descending paper chromatography in the organic phase of toluene:ethyl acetate:methanol:water = 9:1:5:5 or 10:1:5:5, at 37°C (Bush, 1952), and used ethylenediamine to estimate the separated acetylated catecholamines. This technique appeared to be a promising alternative to the phenol:hydrochloric acid method, which, as already pointed out in the preceding section (p. 89, 90), had proved rather unsatisfactory. The apparent advantages of the method involving the acetyl derivatives were as follows:

- (i) the acetylated derivatives of adrenaline, noradrenaline, dopamine, metanephrine, normetanephrine and methoxydopamine could be separated from one another on one paper chromatogram;
- (ii) the absence of phenol in the chromatographic procedure eliminated the difficulties experienced with the amine assays as a result of the presence of phenol

in the chromatogram eluates;

(iii) possible losses due to oxidation of the catecholamines during chromatography would be decreased since they would be in the form of the less readily oxidisable acetyl derivatives;

(iv) extraction of the acetylated amines into an organic solvent from the brain extract might be expected to exclude many substances which contribute to the blank fluorescence and to the bulk of the extract for chromatography, thus reducing the blank and increasing the ease with which the extract can be applied to the chromatogram.

With these points in mind, together with the possibility of estimating the acetylated methoxyamines, in addition to the acetylated catecholamines (Goldstein et al., 1959a), it was decided to investigate the methodology of acetylation. The aim was to develop a method whereby the amines and their carboxylic acid metabolites could be measured in the same brain sample. Initial investigations of this technique were carried out in pure solution. These were followed by development of the method using whole rat brain, while bearing in mind its future application to discrete areas of dog brain (Section II).



Extraction of Dopa, the Catecholamines and their Amine  
and Acid Metabolites, from Rat Brain

The extraction technique was based on that described by Bertler et al. (1958).

Three adult white Wistar rats, weighing 100 - 150 g were killed by decapitation. The brains were removed as quickly as possible and, after discarding the cerebellum, were wrapped in aluminium foil and quickly frozen in solid carbon dioxide contained in a vacuum jar. It was found that they could be stored overnight in this state without any alteration in the concentrations of the catecholamines. The brains were weighed and while still frozen, homogenised in 0.4N perchloric acid at 4°C, 4 ml/g brain tissue, in an all-glass homogeniser. The perchloric acid and the homogeniser had previously been cooled to 4°C and the homogenisation was continued for not more than 3 min. in order to maintain a low temperature during this procedure. Disodium edetate, 2 mg/g brain tissue, and in later experiments, ascorbic acid, 500 µg/g brain tissue, were present during this homogenisation. After the volume had been noted the homogenate was divided into three equal portions. Two of these portions served as duplicate samples for the estimations of the endogenous amines. To the third portion was added a mixture

containing a known amount (200 - 800  $\mu\text{g}$ ) of each of noradrenaline, adrenaline, dopamine, normetanephrine, metanephrine, methoxydopamine, dopa and homovanillic acid. The purpose of the addition of these exogenous substances was to permit the determination of the recovery of each substance through the analytical procedure. These three samples from the same brain homogenate were then processed in parallel. Each sample was chilled to  $4^{\circ}\text{C}$  and centrifuged for 7 min. at 4,300 g. The supernatant was removed and the protein precipitate re-extracted by homogenisation for about 1 min. at  $4^{\circ}\text{C}$  in 0.4N perchloric acid, 3 ml/g of original brain tissue. The two perchloric acid extracts from each sample were combined. Bertler et al. (1958) found that 97% of the catecholamines in brain tissue were extracted into 0.4N perchloric acid under such conditions. In agreement with their findings on the distribution of amines between supernatant and precipitate (p.9 ), the amine values obtained by taking the first supernatant and making an aliquot correction for amine remaining in the precipitate were the same as those obtained by re-extracting this precipitate and combining the two extracts. Re-extraction was necessary in order to obtain sufficient amounts for estimation of those amines such as adrenaline and normetanephrine

which are present in rat brain in very low concentrations (Anton and Sayre, 1964; Carlsson and Lindquist, 1962).

Perchlorate was precipitated from the extract as the potassium salt, by the dropwise addition, with continuous shaking, of 5N potassium carbonate, followed by 1N potassium carbonate when the end-point was neared, until the solution had a pH of 3 - 4. One drop of 0.02% (w/v) bromophenol blue (B.D.H. Laboratory Reagent) served as internal indicator, the end-point of the carbonate addition being indicated by the appearance of a faint grey-blue colour in the solution when viewed through its depth against a white background. In later experiments 5N and 1N potassium hydroxide were used to adjust the pH since they avoided the evolution of carbon dioxide which proved troublesome on occasions. The sample was stored at  $-20^{\circ}\text{C}$  for at least 30 min. to allow maximum precipitation of potassium perchlorate and aggregation of any unprecipitated proteinaceous matter. The sample could be stored overnight at  $-20^{\circ}\text{C}$  without any deleterious effects. After this time, the sample was thawed by immersion in a beaker of cold ( $10^{\circ}\text{C}$ ) water and centrifuged for 7 min. at 4,300 g at  $40^{\circ}\text{C}$ . The supernatant (6 - 7 ml) was transferred into a glass-stoppered test-tube of 35 ml capacity (Quickfit and Quartz, B24). The precipitate was washed with

0.5 ml water at 4°C, centrifuged as before and this wash fluid added to the supernatant.

Removal of carboxylic acid metabolites. In order to remove the carboxylic acid metabolites of the catecholamines from the extract, the supernatant was acidified to a pH between 1 and 2 (Universal Indicator paper) by the addition of 1 - 2 drops of 2N hydrochloric acid, saturated with sodium chloride and shaken vigorously for 4 min. with 1.5 vol. ethyl acetate. The extraction was repeated once. The ethyl acetate layers were separated by centrifugation for 3 - 5 min. at 980 g. The acids were back-extracted into 4.0 ml 0.1M 2-amino-2-(hydroxy-methyl)-propane-1:3:diol (B.D.H. Laboratory Reagent) 'tris' buffer pH 8.6. Homovanillic acid was estimated in the buffer extract by the method Andén, Roos and Werdinius (1963).

Preparation of sample for acetylation. After the removal of the acids, the sample was evacuated at the vacuum pump to remove traces of ethyl acetate. The pH was brought to about 4 with a few drops of 1N potassium carbonate or potassium hydroxide and the sample briefly centrifuged to bring down excess salt (p. 100). At this stage the sample could, if necessary, be stored at - 20°C overnight. The supernatant was taken off, using a 5 ml safety pipette and the precipitate washed briefly with 0.5 ml water at 4°C. If

there was a large amount of sedimented salt and a relatively small supernatant volume, the wash volume was increased to 1.0 - 1.5 ml. This solution (about 6 - 8 ml), containing the amino acids and the amines was acetylated as follows.

Acetylation Procedure (Welsh, 1952)

To the solution was added acetic anhydride (0.05 ml/ml solution) followed immediately by sodium bicarbonate (0.1 g/ml solution). The solution was stirred vigorously for 10 min. before repeating the additions of acetic anhydride and sodium bicarbonate. The stirring was continued for a further 20 min. by which time the evolution of carbon dioxide had ceased and some sodium bicarbonate remained unreacted. The acetylated amines were extracted by shaking the solution three times for 4 min. each time, with 2 vol. dichloromethane. Centrifugation for 3 - 5 min. at 980 g was necessary to break the resultant emulsion. The combined dichloromethane extract was shaken briefly with one third its volume of 0.1N hydrochloric acid to remove any unacetylated amines and then 4 times with one third volumes of water to remove the acid. The dichloromethane extract was dried by adding anhydrous sodium sulphate until there was some powdery, unhydrated salt, in addition to the lumpy, hydrated salt, at the

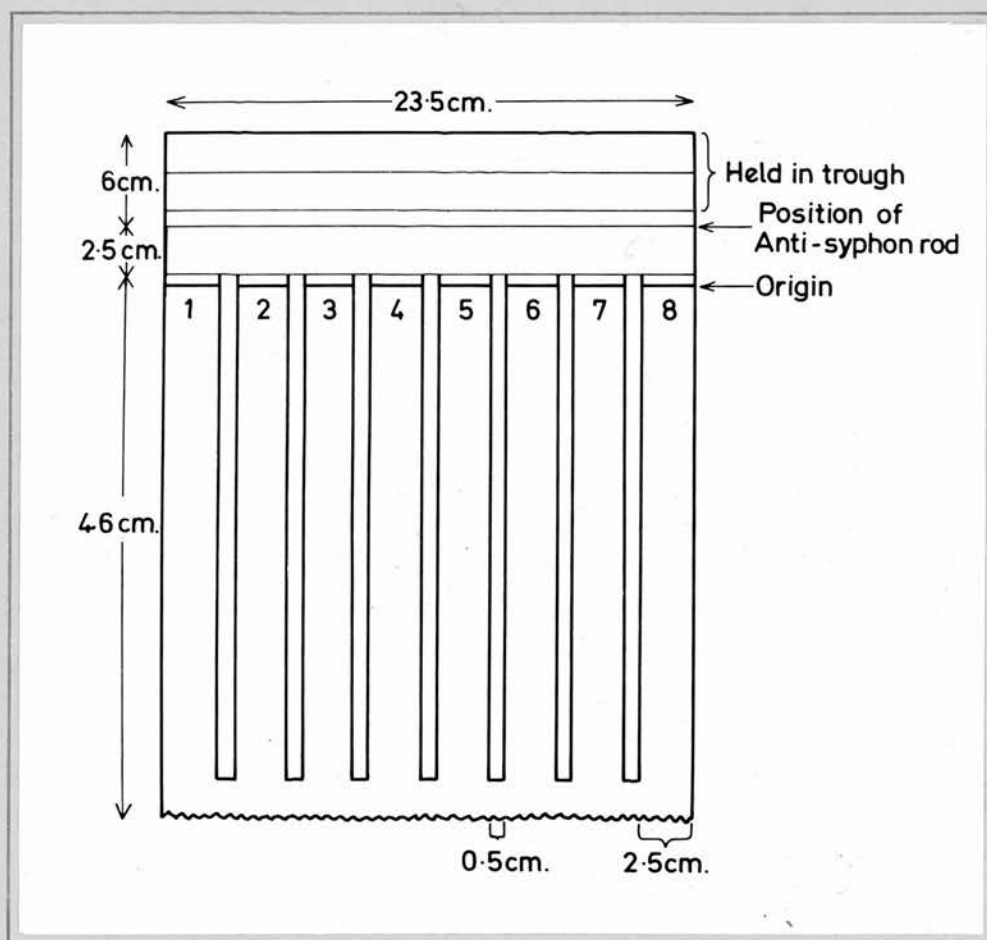
foot of the test-tube. The extract was usually left overnight at this stage, at  $-20^{\circ}\text{C}$ .

The dichloromethane extract was decanted into a 100 ml round-bottomed flask. The sodium sulphate residue was shaken briefly with a further 5 - 10 ml dichloromethane and the organic liquid carefully decanted and combined with the main dichloromethane extract. The extract was evaporated to about 1 ml under a jet of nitrogen at room temperature. The sides of the flask were then carefully washed down with 2 ml dichloromethane and the evaporation continued to dryness. The almost invisible residue containing the acetylated amines was taken up in 0.3 ml followed by 0.2 ml methanol by carefully allowing the methanol to run down the full circumference of the inside of the flask. This was necessary to dissolve any amines which, despite washing down with dichloromethane, remained on the wall of the flask. Swirling of the methanolic extract around the flask was avoided because such a procedure tended to redeposit the amines on the flask wall. Methanol was used because it was found to be an even better solvent for the acetylated amines than dichloromethane.

Comment on acetylation procedure. The solution for acetylation was saturated with sodium chloride

Figure 10

Diagram of Whatman no. 20 Paper Sheet used for  
Chromatography of the Acetylated Amines in Toluene: Ethyl  
acetate: Methanol: Water = 10: 1: 5: 5.





remaining from the acid extraction stage (p.97), so that after addition of sodium bicarbonate during the acetylation stage, a heavy salt deposit was formed. If excess sodium chloride was not removed by centrifugation as described on p.97, this salt deposit was extremely bulky and made removal by pipette, of the dichloromethane phase, which was at the foot of the test-tube along with the salt, very difficult.

Acetyl-dopa. The aqueous phase remaining from the acetylation procedure after extraction of the acetylated amines into dichloromethane, was retained for the estimation of acetyl-dopa (p.118 ).

#### Paper Chromatography of Acetylated Amines

A sheet of Whatman no. 20 paper for chromatography was cut into eight strips, 46 cm long and 2.5 cm wide, the tops and bottoms of neighbouring strips remaining joined by cross-pieces of paper (Figure 10 ). A solution for chromatography was applied to one of the strips at the point marked 'origin' in Figure 10 , by replicate applications from a capillary tube across the full width of the strip. Evaporation of the solvent was hastened by projecting a continuous stream of nitrogen from a manifold onto the paper strips at the sites of application of the solutions.

Care was taken that an applied solution formed a band not exceeding 1 cm in width at any time during application to the paper.

To each of the origins of strips 1, 3, 5 and 7 was applied a mixture of the acetyl derivatives of adrenaline, noradrenaline, dopamine, metanephrine and normetanephrine equivalent to about 20  $\mu$ g of each amine and acetyl-methoxy-dopamine equivalent to about 30  $\mu$ g unacetylated amine. These amounts could be adequately visualised on the developed chromatograms, and served as markers for localisation of the acetylated amines in the 'sample' chromatograms. These marker mixtures were prepared from solutions in 50% (v/v) methanol of each acetylated amine, containing the equivalent of 10 mg/ml of the parent amine (p. 108). To the origins of strips 2 and 6 were applied the methanolic solutions containing the acetylated amines from the rat brain extracts. To the origin of strip 4 was applied the similar solution from the rat brain extract to which known amounts of the amines had been added (p. 95). These extracts, equivalent to 1.0 - 1.5 g brain tissue were easily applied to the paper and produced a barely detectable deposit on application. Nothing was applied to the origin position in strip 8. This strip was used for the preparation of paper blanks required for the fluorimetric assay of each amine (p. 106).

An all-glass 'Panglass' (Shandon, 30 x 20 x 56 cm) chromatography tank was lined with 2 thicknesses of filter paper impregnated with and dipping into, a layer of the developing solvent in the foot of the tank. The chromatogram carrying the samples and markers was suspended between one long side of the tank and a filter paper curtain saturated with the aqueous phase. This curtain was held in a trough containing the aqueous phase, placed across the length of the top of the tank. Saturation of the tank with vapour from the aqueous phase was maintained by the continual descending flow of the aqueous phase, down the filter-paper curtain, into a receiving trough at the foot of the tank. The chromatograms were allowed to equilibrate for at least 6 hr. at 19 - 22°C in a thermostatically controlled room. At the end of the equilibration period, 100 ml of the organic phase of a mixture of toluene:ethyl acetate:methanol:water (10:1:5:5 by vol.) was introduced into the solvent trough and the descending chromatograms allowed to develop for 10½ - 11 hr. at 19 - 22°C. In order to obtain maximum separation of acetyl-noradrenaline from acetyl-normetanephine, the time and temperature of development were adjusted so that acetyl-methoxydopamine, the acetylated amine with the greatest Rf, was close to the foot of the paper strips.

Freshly distilled solvents were used for the preparation of the developing solvent system. Tanks with polythene accessories and plugs were unsatisfactory, because polythene absorbed the solvent vapours causing leakage. A tight seal between the lid and walls of the tank and between the lid and its coverslip was essential and was achieved using starch-glycerin paste (p. 33 ). Screw clamps, one at each end of the tank, were necessary to ensure firm apposition of the tank-lid on top of the tank walls. The system was extremely temperature dependent. The tank was kept, raised off the floor, in a large box made of 2 inch polystyrene with a 3 inch air-space between tank and box, in order to protect the contents of the tank from draughts and ensure an even temperature within the tank. The slightest temperature gradient across the length of the tank resulted in differences in rates of flow of the developing solvent down the chromatogram strips exposed to the different temperatures, with consequent differences in the distances travelled by a given acetylated amine on these chromatogram strips.

After development, the chromatogram was dried by hanging it in a chromatography tank through which was passed a brisk stream of nitrogen. The strips bearing the marker acetylated amines were cut out and

the amines were visualised by spraying the strips with a solution of diazotised p-nitroaniline. After the paper had dried it was sprayed with 5% sodium hydroxide in 50% (v/v) methanol. Fresh diazotised p-nitroaniline was prepared on each occasion by mixing 20 ml of a solution of 0.5% (w/v) p-nitroaniline (B.D.H. Laboratory Reagent) in approx. 0.8N hydrochloric acid (8 ml conc. hydrochloric acid in 100 ml water) with 1.2 ml 5% (w/v) sodium nitrite. The acetylated amines were eluted from the appropriate parts of the sample strips and the blank strip (p.102) in 15 ml glass-stoppered test-tubes by gentle shaking for 30 min. at room temperature in the following solutions: acetyl-adrenaline, acetyl-noradrenaline and acetyl-dopamine in 3.00 ml water; acetyl-metanephrine and acetyl-normetanephrine in 2.00 ml 0.4N hydrochloric acid and acetyl-methoxydopamine in 2.00 ml 0.1N hydrochloric acid. After 30 min., the papers were drawn up the sides of the tubes to drain and the eluates stored at 4°C until assayed. The acetylated catecholamines were estimated within 24 hr. and the acetylated methoxyamines within 4 days, of their elution.

#### Blanks

Since there is no obvious way of preparing a

tissue blank during the method of assay using ethyldiamine it is necessary to have an extract blank for these assays. Two methods of preparing an extract blank were investigated and compared. In the first method, rat cerebellum was extracted, acetylated and chromatographed in parallel with the rat brain samples (p. 94-105). It was presumed that rat cerebellum contains negligible amounts of the catecholamines, since Juorio and Vogt (1965) found no homovanillic acid in rat cerebellum. In the second method, a paper strip with nothing applied to it, was included in the chromatograms (p. 102). Blanks were prepared for each amine by cutting the cerebellum and the blank chromatogram strips at the same positions as the sample strip and eluting and assaying as for each acetylated amine. The paper blanks were judged to be satisfactory and superior to the cerebellum blanks, for the following reasons:

- (i) In the case of the 'acetyl-normetanephrine' assay, (in which it is possible to prepare a faded blank (p. 116), the paper blank gave the same reading as the faded blank and the cerebellum blank a slightly higher reading.
- (ii) Under normal conditions, adrenaline and metanephrine are not detectable in rat brain. As expected, the fluorescence readings obtained for these

amines in brain chromatogram eluates were the same as those obtained from paper chromatogram eluates. The fluorescence readings from the cerebellum chromatogram eluates were, however, slightly higher than those from the brain chromatogram eluates.

(iii) Those parts of a brain chromatogram not corresponding to any amine gave the same fluorescence as their paper blank but again, the fluorescence from the cerebellum extract was slightly higher.

After these experiments had been carried out, Glowinski and Iversen (1966) published an estimate for noradrenaline in rat cerebellum of 0.17  $\mu\text{g/g}$ . The presence of noradrenaline in this amount and possibly of other catecholamines and their amine metabolites in rat cerebellum, could account for the cerebellum chromatogram eluates giving rise to a greater fluorescence than the paper eluates.

#### Assays of Acetylated Amines and Dopa

##### Preparation of Standard Solutions of the Acetyl Derivatives of Adrenaline, Noradrenaline, Dopamine, Metanephrine, Normetanephrine, Methoxydopamine and Dopa

20  $\mu\text{g}$  of the substance contained in 5 ml 0.005N hydrochloric acid was acetylated according to the procedure described on p. 98. The residue from the evaporated dichloromethane extract (or the ethyl



acetate extract in the case of dopa (p. 118) was dissolved in 10 ml de-ionised distilled water. The solution so obtained, containing the equivalent of 2 ug of the parent substance/ml, was stored in a 15 ml glass-stoppered test-tube at 4°C. This solution was used as a standard solution in the course of the estimation of the parent substances in 'unknown' solutions of the acetyl derivatives. These standards were prepared at monthly intervals and a new preparation was checked against the previous preparation before being used as a standard for assays. Standards prepared at monthly intervals over a period of 5 months gave the same readings when assayed together, demonstrating the reproducibility of the acetylation technique and the stability of the standard solutions, as judged by the fluorescence yield, over a period of several months when stored at 4°C.

Solutions of the acetylated amines for use as markers were prepared in the same way except that 15 mg of the substance was acetylated and the residue taken up in 1.5 ml 50% (v/v) methanol to give a solution containing the equivalent of 10 mg/ml unacetylated amine.

Estimation of Acetyl-adrenaline, Acetyl-noradrenaline and Acetyl-dopamine

These compounds could be assayed by simultaneous

hydrolysis and coupling with ethylenediamine to form their respective fluorophors (p. 22). To a 2.00 ml sample containing the acetylated amine, in a 15 ml test-tube with ground glass stopper, was added 0.50 ml of a freshly prepared and cooled mixture of ethylenediamine and 4M ammonium chloride in the proportions of 1:1.3 by vol. (Kägi, Burger and Giger, 1957). The sample was heated at 50°C for 20 min. with shaking, in a covered water-bath and then cooled. It was saturated with sodium chloride and the fluorophor extracted into 2.00 ml isobutanol (Weil-Malherbe, 1959) by shaking for 4 min.. The phases separated in 2 - 3 min. The isobutanol layer was then withdrawn with a safety pipette and the relative fluorescence measured on the Aminco-Bowman SPF at 430/500 mμ for acetyl-noradrenaline and acetyl-adrenaline and at 430/520 mμ for acetyl-dopamine. The relative fluorescences of reagent blanks and aqueous standard solutions of the acetylated amines were determined concurrently with each batch of samples. Internal standards proved unnecessary since eluates from brain chromatograms were found to have no effect on the fluorophors from the acetylated amine standards.

The isobutanol used in these assays was obtained from British Drug Houses and was of the grade 'recommended for fluorimetric assay' (p. 33 ). A

fresh bottle was used every 2 weeks. The blank obtained from such isobutanol was low and was not lowered further by distillation. Old stocks of isobutanol, on the other hand, gave very high blanks, which could be reduced by 1 - 3 distillations. Freshly distilled, absolutely colourless, ethylenediamine, prepared at 2 week intervals as described on p. 32, was essential. Ethylenediamine stored for longer than 2 weeks tended to produce high blanks, even though it remained apparently colourless.

#### Estimation of Acetyl-methoxydopamine

Acetyl-methoxydopamine cannot be simultaneously oxidised to the corresponding quinone and coupled with ethylenediamine as can acetyl-dopamine, because the methoxy group is resistant to oxidation by alkali. It was found however, that the O-acetyl group of acetyl-methoxydopamine could be removed by acid hydrolysis leaving the methyl monoether which, in accord with Adler and Magnusson's (1959) report, could be oxidatively demethylated to the free catechol by treatment with periodate. Fluorophor formation from the liberated catechol by reaction with ethylenediamine was then possible.

A 1.00 ml portion of a solution of acetyl-methoxydopamine in 0.1N hydrochloric acid (standard solution or chromatogram eluate), contained in a glass-stoppered 15 ml test-tube was heated for 20 min. in a boiling water-bath. After cooling to room temperature, 0.50 ml 0.1% (w/v) sodium periodate was added to the solution and the reaction allowed to proceed for 5 min. under subdued lighting conditions. Ethylenediamine-ammonium chloride mixture (p.109) 0.50 ml, was then introduced into the solution and the fluorophor developed and extracted into isobutanol, as described for the estimation of the acetylated catecholamines (p.109). Although the maximum fluorescence occurred at 430/520 m $\mu$ , the relative fluorescence was measured at 440/540 m $\mu$  because the blank contributed proportionally less to the fluorescence at the latter wavelengths than it did at the former. Reagent blanks and aqueous acetyl-methoxydopamine standards were run in parallel with each batch of samples. Internal standards proved to be unnecessary since eluates from brain chromatograms had no effect on the fluorescence from acetyl-methoxydopamine.

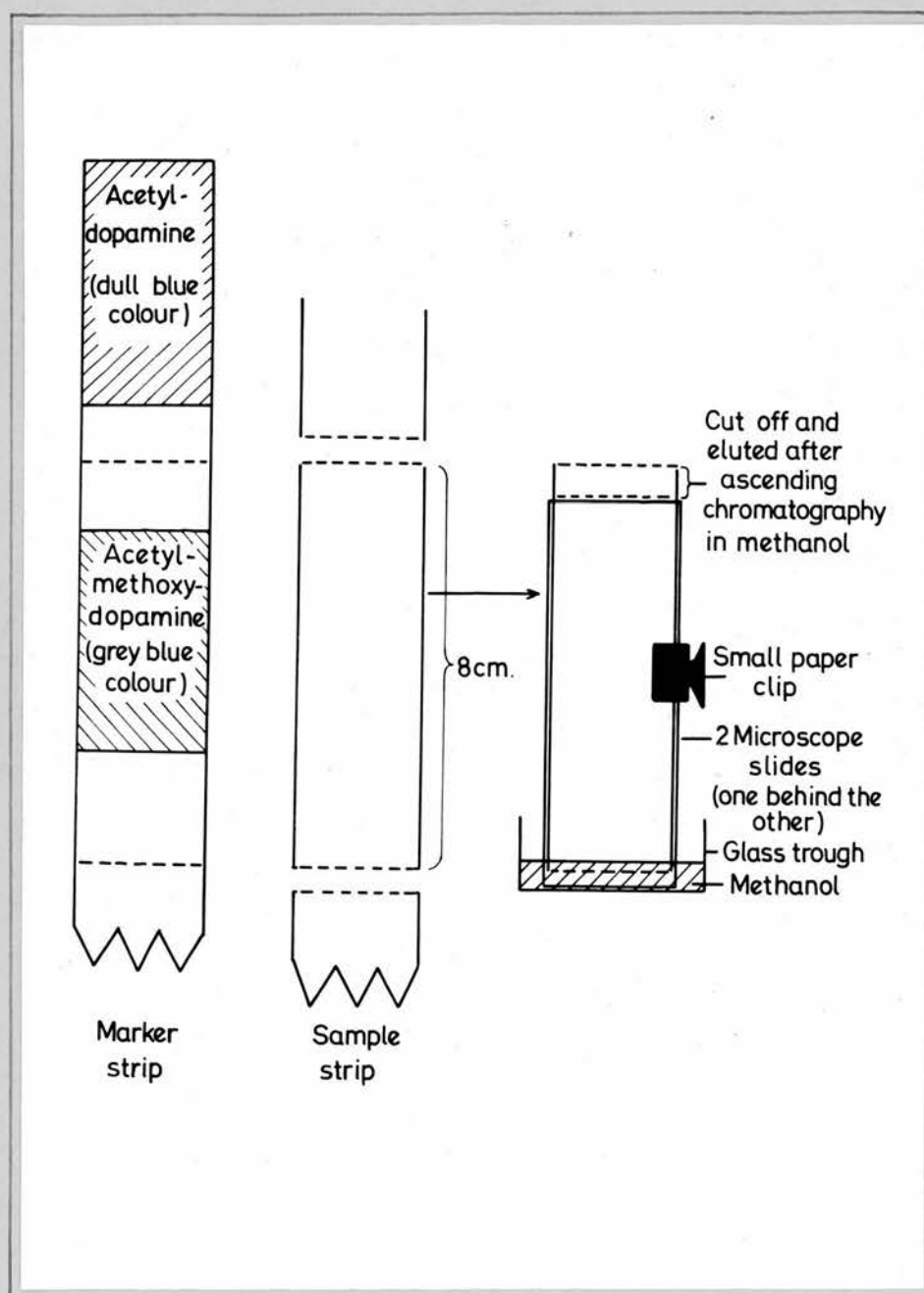
The 0.1N hydrochloric acid paper eluates gave fluorescence readings 3 to 5 times those from the reagent blanks. The cause of these high paper blanks

was some material which was leached out of the paper by water (acid conditions increased the amount of, but were not essential for, this leaching effect) and which reacted with periodate in acid solution to produce a fluorescence on addition of the alkaline ethylenediamine mixture. Pre-washing the chromatography paper in a 50:50 (by vol.) benzene:methanol mixture, as advocated by Goldstein, Friedhoff, Simmons and Prochoroff (1960) did not remove this interfering material. Pre-washing the chromatography paper in 0.1N hydrochloric acid followed by water was abandoned because washing the paper free of traces of acid was difficult and the use of an acidic paper resulted in some hydrolysis of the acetylated amines to their partially acetylated derivatives which failed to move from the origin during the chromatography.

A reduction of the paper blank to that obtained from ethylenediamine treated eluates of the acetyl-catecholamine portions of the chromatogram was achieved by concentrating the acetyl-methoxydopamine by ascending chromatography in methanol, into a much smaller area of the paper segment before elution. The 'acetyl-methoxydopamine' section of the sample or blank chromatogram was sandwiched between two microscope slides with the end proximal to the origin of the

Figure 11

Technique for Concentrating Acetyl-methoxydopamine spread over 3 - 5 cm length of a Chromatogram Strip, into a 0.7 cm length of Chromatogram Strip.



chromatogram projecting 0.7 cm from the top of the slides (Figure 11 ). The bottom of the slides was inserted into a glass trough containing methanol so that the lower end of the paper strip was in line with the surface of the solvent. The solvent flowing up the paper evaporated from the exposed part of the strip but eventually reached the upper edge, when the flow was allowed to continue for a further 6 min.. The strip was removed and allowed to dry and the top 0.7 cm eluted in 2.00 ml 0.1N hydrochloric acid. No acetyl-methoxydopamine was detected in the remainder of the strip in a control experiment in which the acetyl-methoxydopamine section of a chromatogram of a brain extract to which had been added 400  $\mu$ g acetyl-methoxydopamine was subjected to this concentrating technique, thus demonstrating its efficiency at the  $\mu$ g level.

#### Estimation of Acetyl-normetanephine

Acetyl-normetanephine after acid hydrolysis and treatment with periodate might be expected to yield, on coupling with ethylenediamine, a fluorophor having the same characteristics as that formed from acetyl-noradrenaline. In fact, the sensitivities of assay of normetanephine and acetyl-normetanephine



by this method were only 200  $\mu\text{g}$  and 300  $\mu\text{g}$ , respectively i.e. considerably less than that of acetyl-noradrenaline. Sensitivity was greatly increased if an adaptation of Brunjes' (1964, p. 59) trihydroxy-indole method, involving an essential initial hydrolysis of the O-acetyl ester, was used. Hydrolysis in 0.4N hydrochloric acid gave the maximum reading for the fluorescence; increasing the concentration of hydrochloric acid above 0.4N did not increase the fluorescence yield.

1.00 ml 0.4N hydrochloric acid paper eluate in a 15 ml glass-stoppered test-tube was heated for 20 min. in a boiling water-bath and cooled thoroughly. During the subsequent development of the fluorophor, the sample was kept in the dark by insertion in the solid wooden test-tube rack used in Section I, Sub-section 1 (p. 48). This precaution was taken since the trihydroxyindole fluorophor derivative of normetanephrine was found to be light sensitive (p. 59). To the solution was added 0.50 ml zinc sulphate (0.5% (w/v)  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ) in 0.02N hydrochloric acid, followed by 0.50 ml 0.075% (w/v) potassium ferricyanide (freshly prepared from a 0.25% (w/v) stock solution, stored in a dark bottle at  $4^\circ\text{C}$ ). Exactly 4 min. after the ferricyanide addition 0.7 ml 1M sodium acetate solution previously adjusted to pH about 12.9 with 5N

sodium hydroxide was added to bring the pH of the solution to 5.5. After a further 4 min. interval, 0.50 ml of a freshly prepared mixture containing 8.70 ml 5N sodium hydroxide, 0.30 ml redistilled ethylenediamine and 1.00 ml freshly prepared 2% (w/v) ascorbic acid, was added. The solution was thoroughly mixed after each reagent addition. The relative fluorescence was read in the Aminco-Bowman SPF at 400/500 m $\mu$ , 20 - 30 min. after addition of the ferricyanide, to allow time for maximum production of the fluorophor.

Unlike in the ethylenediamine method, it is possible in the above method, to obtain a faded blank which can be used as a tissue blank, by adding only the sodium hydroxide (0.44 ml 5N) at 4 min. and waiting a further 10 min. before adding 0.06 ml of a freshly prepared mixture of ethylenediamine (0.30 ml) and ascorbic acid (1.00 ml of a 2% solution). This faded blank gave the same fluorescence reading as the chromatogram paper blank. The latter blank was used routinely because it was technically simpler and because, unlike the faded blank it did not utilise a portion of the sample chromatogram eluate and thus left all the acetyl-normetanephine in the eluate available for assay. Reagent blanks and aqueous standards were processed along with each batch of samples. It was found possible for one person to cope

with a maximum of 8 samples, including blanks and standards, in any one run. Internal standards were not processed routinely, since it was found that they did not differ significantly from aqueous standards and because, as was also the case in the other assays (p.108,110), it was essential to conserve the available acetylated amine for the estimation in view of the very small amounts present.

#### Estimation of Acetyl-metanephrine

Although the sensitivity of estimation of metanephrine after treatment with periodate and ethylenediamine as described for acetyl-normetanephrine (p. 114) was 20 mpg, the sensitivity of assay of acetyl-metanephrine (obtained by this method) after acid hydrolysis, was only 200 mpg. Acetyl-metanephrine could be measured with a sensitivity of 10 mpg by the method used to estimate acetyl-normetanephrine (p. 115). Addition of sodium acetate was omitted since oxidation of hydrolysed acetyl-metanephrine, unlike oxidation of hydrolysed acetyl-normetanephrine, proceeded satisfactorily in 0.4N hydrochloric acid. The acetyl-metanephrine fluorophor was more rapidly produced and broken down than the acetyl-normetanephrine fluorophor. The fluorescence derived from acetyl-metanephrine, at 420/520 mμ, was therefore measured

about 15 min. after the addition of the ferricyanide. Reagent blanks, aqueous standards, faded blanks and internal standards were as for acetyl-normetanephrene.

#### Extraction and Estimation of Acetyl-dopa

The aqueous phase remaining from the acetylation procedure after extraction with dichloromethane (p. 101 ) would contain acetyl-dopa and any other acetylated amino acids, such as acetyl-tyrosine. Acetyl-dopa could be assayed in this aqueous phase but such a procedure would not be advisable if this aqueous phase was derived from a tissue extract, in which tissue substances liable to give rise to high blanks and/or quenching may be present. Further purification of the acetyl-dopa-containing solution before assay is therefore desirable. Purification was carried out in the following way. The pH of the solution was adjusted to about 2 (B.D.H. wide range indicator paper) by the dropwise addition of conc. hydrochloric acid. It was found that for each ml acetic anhydride used in the acetylation procedure, about 1.8 ml conc. hydrochloric acid was required for this pH adjustment. The solution was still saturated with sodium chloride from the acid extraction stage (p. 97 ) during the subsequent extraction of the acetyl-dopa into ethyl acetate. Two extractions, with

2 vol. of the solvent, with shaking for 4 min. each time, were carried out, separation of the phases being aided by centrifugation (2 - 3 min. at 980 g). The combined ethyl acetate extracts were dried by the addition of the minimum amount of anhydrous sodium sulphate for at least 1 hr. at 4°C. The extract was sometimes stored overnight at this stage, at -20°C. It was then decanted from the sodium sulphate through filter paper (Whatman no. 1) and evaporated to dryness under a jet of nitrogen at room temperature. The very faint, slightly greasy residue, tinged with varying amounts of a whitish powder, presumably traces of sodium sulphate, was dissolved in 3.00 ml water and assayed for acetyl-dopa as described on p.120.

An alternative procedure of back-extraction of acetyl-dopa from the ethyl acetate extract into an alkaline buffer was possible, but not technically feasible, on account of the relatively high concentration, in the ethyl acetate, of acetic acid (about 1 ml 10M) liberated by the addition of the conc. hydrochloric acid to the sodium acetate contained in the solution from the acetylation reaction.

Estimation of acetyl-dopa. The same technique used to assay the acetylated catecholamines was suitable for the assay of acetyl-dopa. The

acetyl-dopa fluorophor was not extractable into isobutanol, even after acidification of the solution to pH about 1 + 2. The fluorescence was therefore measured in the aqueous solution, at 430/540 mμ. Internal standards proved necessary when estimating the acetyl-dopa content of extracts from brain since these were found to produce significant quenching of the fluorescence.

INVESTIGATION AND DEVELOPMENT OF METHODS FOR THE ASSAY  
OF A MIXTURE OF THE CATECHOL- AND METHOXY-AMINES  
AND DOPA

Each procedure in the preceding 'Methods' section, with the exception of the perchloric acid extraction, was separately examined in pure solution with the amines and dopa. The perchloric acid extraction was not investigated in pure solution because of difficulties experienced in making the several pH adjustments necessary during this procedure, in the absence of the buffering action of tissue extracts (p. 81 ). The feasibility of combining the acetylation, chromatography and assay stages, was then investigated using a mixture of the amines and dopa in pure solution and any adjustments in technique necessitated by this combination, made. Before application of the entire method to the estimation of the amines and dopa in rat brain, the effect of brain extracts, prepared by this method, on the disposition of several of the acetylated amines on a chromatogram, was investigated.

Effect of Ethyl Acetate Extraction on Adrenaline,  
Noradrenaline and Dopa

The following experiments were carried out to check that the catecholamines and dopa were not



extracted from acid solution into ethyl acetate, along with their carboxylic acid metabolites (p. 97).

Duplicate samples of 200  $\mu$ g adrenaline or of 200  $\mu$ g noradrenaline in 4 ml 0.1N perchloric acid were extracted with 3 x 4 ml ethyl acetate and the amine remaining in the aqueous phase assayed, using Häggendal's method (p. 48), after removal of perchloric acid as its potassium salt. 87% and 91% of the added adrenaline and 86% and 92% of the added noradrenaline was recovered in the aqueous phase, showing that there was no appreciable loss of adrenaline or noradrenaline into ethyl acetate from an acid aqueous solution.

Duplicate samples of 2.5  $\mu$ g dopa in 3 ml water at pH about 2 were saturated with sodium chloride and extracted with 2 x 6 ml ethyl acetate. The bulked ethyl acetate fractions were dried over anhydrous sodium sulphate, decanted, evaporated to dryness under nitrogen, taken up in 3 ml water and assayed for dopa, using ethylenediamine (p. 119). No dopa could be detected, indicating that none had been extracted into ethyl acetate from the acid aqueous solution.

#### Assays of Acetylated Amines and Dopa

A linear relationship was found between the amount of substance present and the fluorescence intensity of

Figure 12

Calibration Curves for Acetyl-metanephrine and  
Metanephrine assayed by the Trihydroxyindole Method (p.117 ).

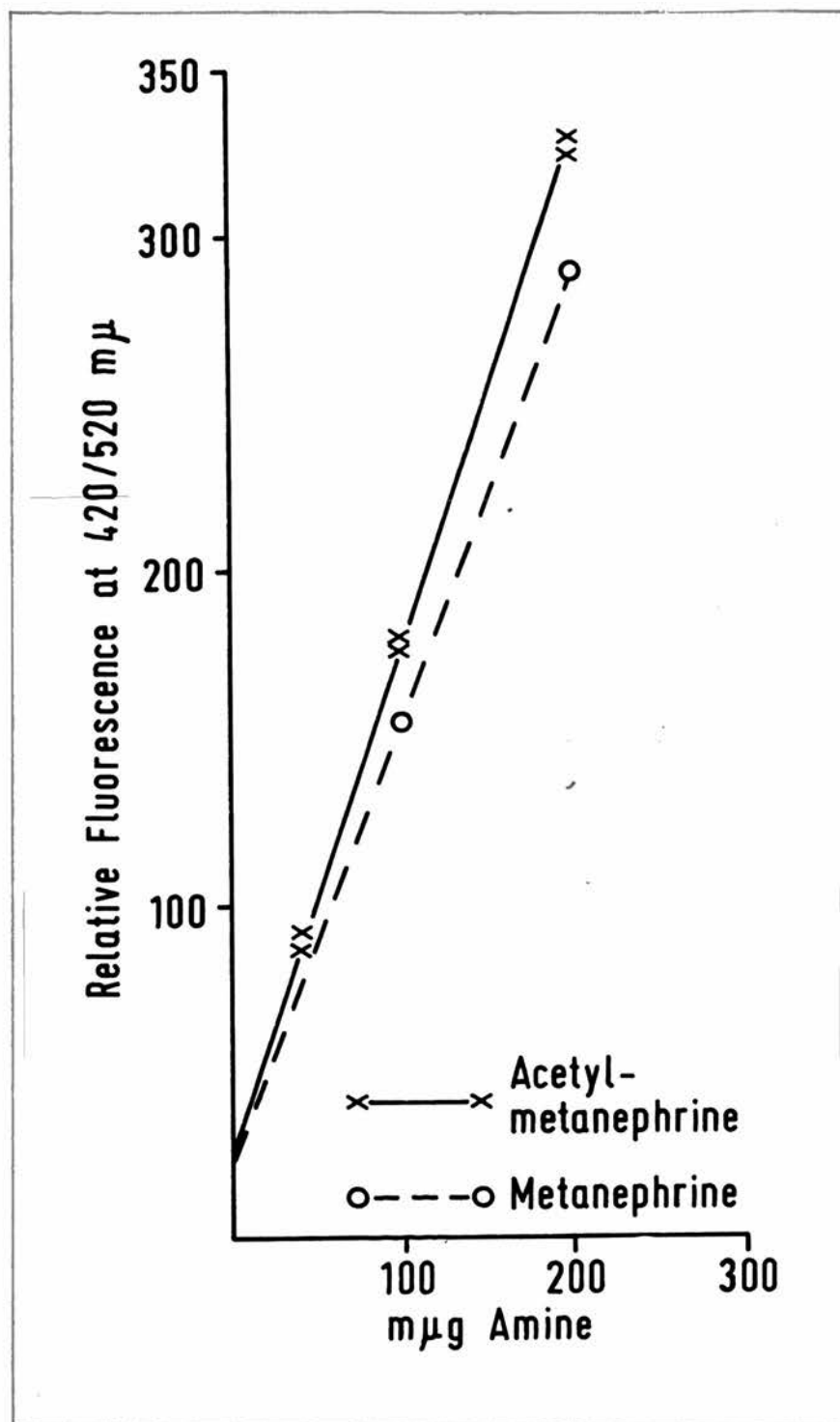


Figure 13

Calibration Curves for:

(1) Acetyl-noradrenaline and Noradrenaline

(2) Acetyl-adrenaline and Adrenaline

assayed by the Ethylenediamine Method (p.108).

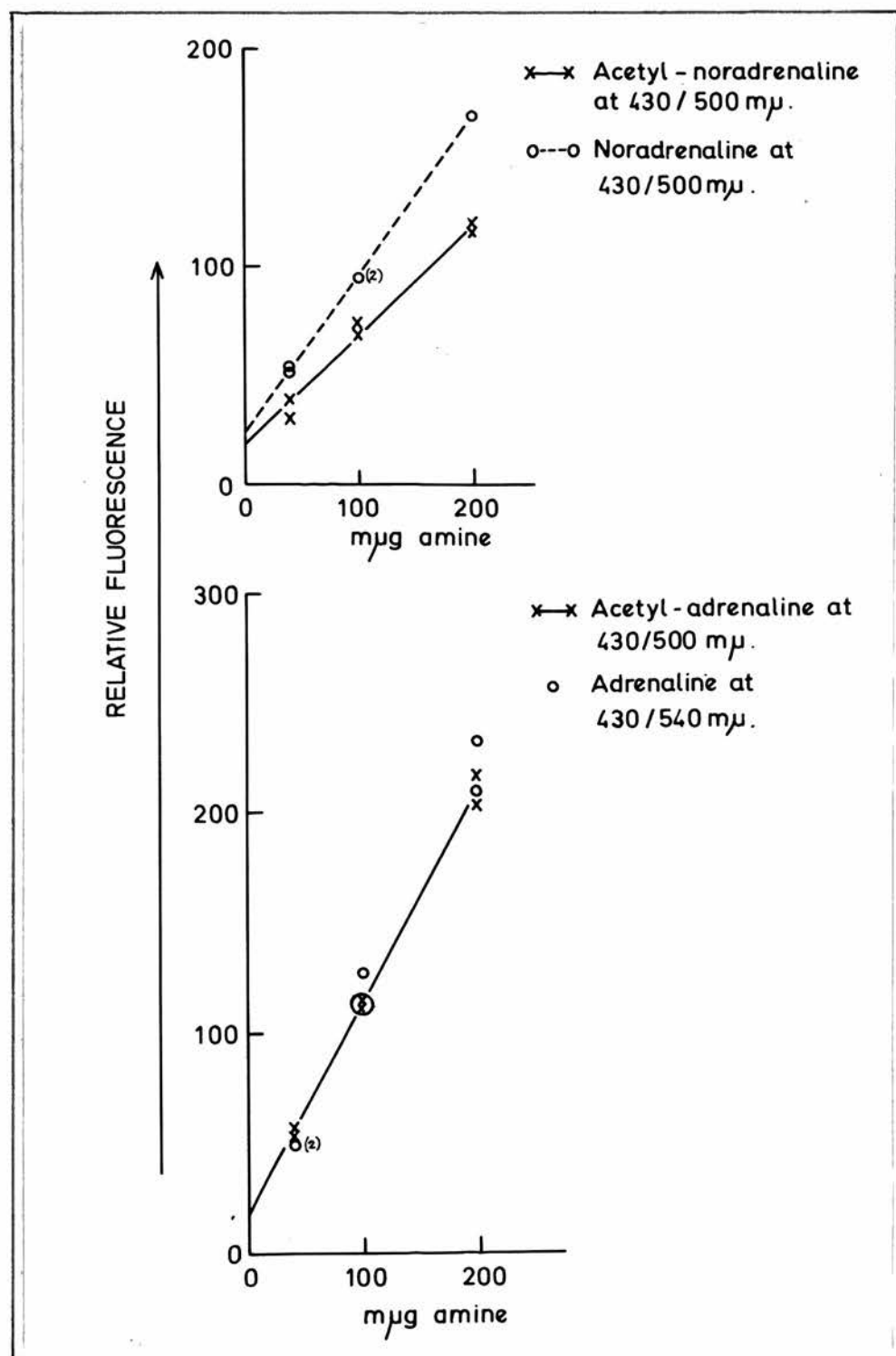


figure 14

Calibration Curves for:

(1) Acetyl-dopamine and Dopamine

(2) Acetyl-methoxydopamine and Methoxydopamine

assayed by the Ethylenediamine Method (p. 110).

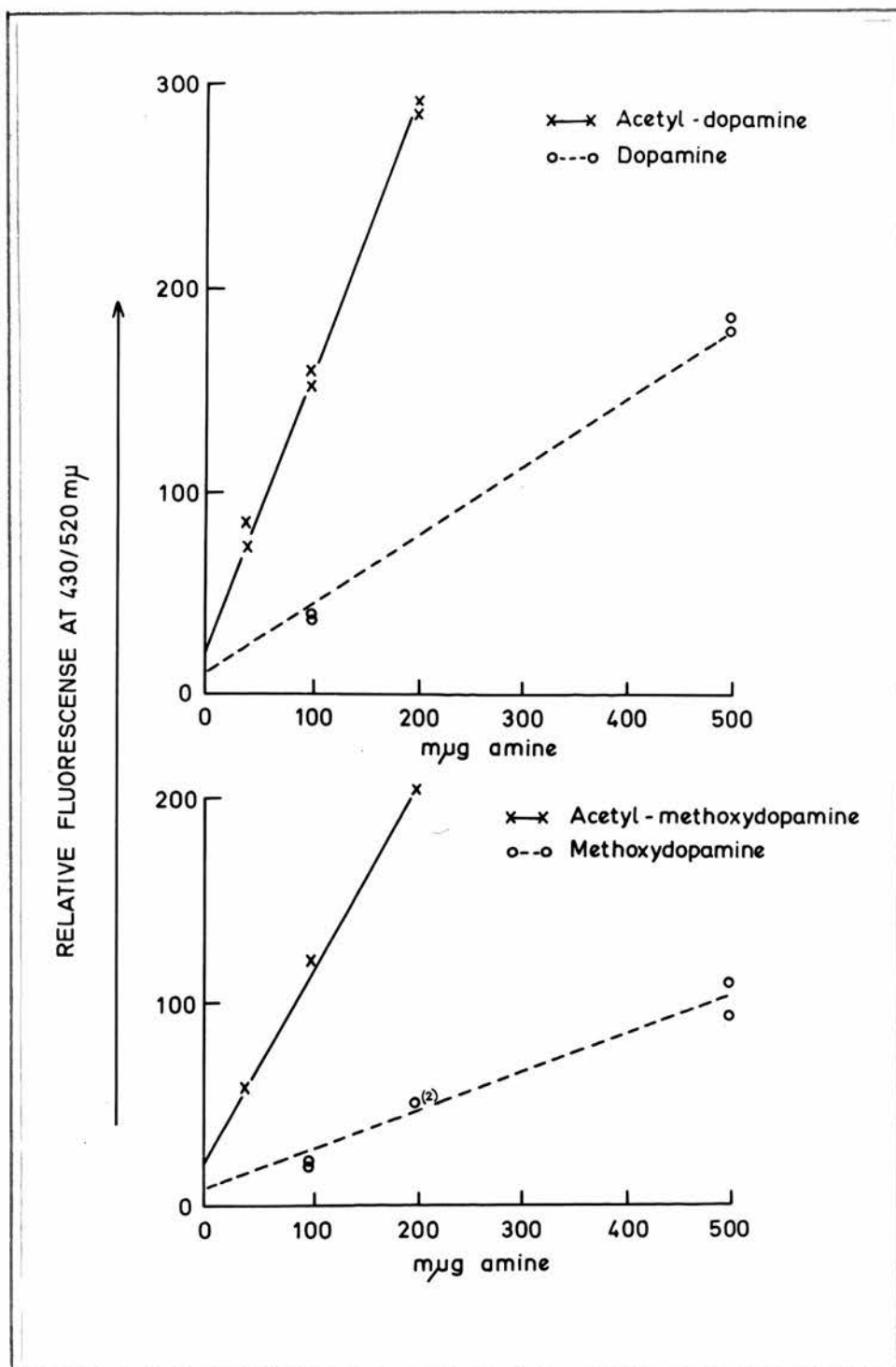


TABLE 8

Fluorescence Characteristics and Sensitivities of Various Assays  
in Pure Solution

	Method of Assay	Fluorescence Peaks (mμ)		Sensitivity of Assay (mμg)		
		Unacet.	Acet.	Unacet.	Acetylated	
					Range (n)	Mean
+ Dopa	E.D.	360/470	430/540	20	30-70 (8)	40
+ Noradrenaline	E.D.	430/500	430/500	15	20-50 (11)	30
Normetanephrine	0.4N HCl + 10 <sup>-4</sup> at pH 5.0 + E.D.	430/490	430/490	200		300
Noradrenaline	0.4N HCl + T.H.1	400/500	420/500	20		45
* Normetanephrine	0.4N HCl + T.H.1	400/500	400/505	20	40-65 (6)	45
+ Adrenaline	E.D.	430/540	430/500	20	10-45 (10)	20
Metanephrine	0.4N HCl + 10 <sup>-4</sup> at pH 4.5 + E.D.	430/540	430/500	20		200
Adrenaline	0.4N HCl + T.H.1	420/520	420/520	10		10
* Metanephrine	0.4N HCl + T.H.1	420/520	420/520	10	5-15 (10)	10
+ Dopamine	E.D.	430/525	530/525	45	5-20 (12)	15
* Methoxydopamine	0.1N HCl + 10 <sup>-4</sup> at pH 1 + E.D.	440/515	430/525	50	10-30 (12)	20
Methoxydopamine	0.4N HCl + Carlsson and Waldeck (1964) (p. 128)	335/385	335/385	100		400

+ and \* indicate methods chosen for routine use

'0.4N HCl' = heating in 0.4N HCl in boiling water-bath for 20 min.

'0.1N HCl' = heating in 0.1N HCl in boiling water-bath for 20 min.

E.D. = ethylenediamine; T.H.1 = trihydroxyindole method, using modification of Brunjes' technique.

Ranges in sensitivity only obtained for those assays used routinely.

Sensitivities calculated as that amount of amine giving the same reading as the reagent blank, at the wavelengths for maximum excitation and emission of the fluorescence.

the fluorophor derived from each of the acetylated amines and dopa when the appropriate assay procedure was examined using pure solutions containing amounts ranging from 40  $\mu\text{g}$  to 200  $\mu\text{g}$  of the substance. Linearity was found to persist with acetyl-dopamine with amounts up to 8.0  $\mu\text{g}$  in the sample. A linear relationship was also found between the amount of substance present and the fluorescence intensity of the fluorophor from each of the unacetylated amines and dopa, when the assay for the corresponding acetylated compound without any hydrolysis was examined in pure solution with 40  $\mu\text{g}$  to 200  $\mu\text{g}$  of the unacetylated amine or dopa (Figures 12, 13, 14 ).

Table 8 summarises the results of the assay investigations and includes assay methods which were not used because of insensitivity or impracticability. All the amines, whether acetylated or unacetylated, yielded fluorescent derivatives on being subjected to the ethylenediamine assay method or the trihydroxy-indole assay procedure. Acetyl-adrenaline and acetyl-noradrenaline could be estimated with approximately equal sensitivity, using either method. For the assay of these substances the ethylenediamine procedure was chosen for routine use because it was technically simpler and the conditions necessary for production of the ethylenediamine fluorophors were found to be less

critical than those required for formation of the trihydroxyindole fluorophors. Acetyl-metanephrine and acetyl-normetanephrine, on treatment with the ethylenediamine reagent, produced fluorophors having only about 10% the intensity of the fluorophor produced by treating the same amount of acetyl-adrenaline with ethylenediamine. Fortunately, the derivatives obtained by treating acetyl-metanephrine and acetyl-normetanephrine according to the trihydroxyindole method had about the same fluorescence intensity as the trihydroxyindole fluorophor from acetyl-adrenaline. The fluorophor obtained by putting acetyl-methoxydopamine through the trihydroxyindole method (Carlsson and Waldeck's method for the estimation of methoxydopamine, 1964) after hydrolysis in 0.4N hydrochloric acid, was only 2.5% the intensity of the trihydroxyindole fluorophor derived from adrenaline. However, the alternative method of estimation, using ethylenediamine, yielded a fluorescent derivative from acetyl-methoxydopamine having about the same fluorescence intensity as the ethylenediamine conjugate from acetyl-adrenaline. No attempt was made to estimate acetyl-dopamine by the trihydroxyindole method, since it was very unlikely, in view of the results obtained on applying this method to the determination of methoxydopamine and its acetylated derivative, that the



trihydroxyindole method would give a more intense fluorescence from acetyl-dopamine, than that produced by coupling acetyl-dopamine with ethylenediamine. Unlike the trihydroxyindole method, there is no way of preparing a tissue blank using the ethylenediamine method. This difficulty was avoided when the ethylenediamine method was applied to chromatogram eluates by using the paper blank (p. 105).

The acetylated catecholamines could be assayed by the methods (marked \* in Table 8) used to estimate their methoxy derivatives but the acetylated methoxyamines could not be assayed by the methods (marked + in Table 8) used to estimate the acetylated catecholamines. Fluorophors with the same wavelength characteristics were obtained on estimating an acetylated catecholamine and its respective methoxy derivative by the method used to estimate the acetylated methoxyamine.

#### Acetyl-methoxydopamine Estimations

There was no significant 'between-duplicate' difference in the fluorescence intensities derived from duplicate samples of different amounts of acetyl-methoxydopamine or of acetyl-dopamine processed concurrently using the 'acetyl-methoxydopamine' method.

However the ratio of the fluorescence intensities derived from equimolar amounts of acetyl-methoxydopamine and acetyl-dopamine using the 'acetyl-methoxydopamine' method of assay, was found to vary from 0.6 to 1.0 on the 12 occasions on which the comparisons were made. This varying ratio was not accompanied on these occasions by a similar variability in the ratio of the fluorescence intensities obtained from acetyl-dopamine when assayed by the 'acetyl-methoxydopamine' and the 'acetyl-dopamine' methods. This latter ratio varied only from 0.9 to 1.0, indicating that the preliminary boiling in 0.1N hydrochloric acid followed by treatment with periodate, had little or no effect on the fluorescence yield from coupling acetyl-dopamine with ethylenediamine. The most probable explanation of the above observation would appear to be a variability in the extent of conversion of the acetyl-methoxydopamine to the catechol derivative, this variation arising from small uncontrollable differences in technique on different occasions. The cause of this variation has not been further investigated, in view of the fact that the variation was not apparent with replicate samples processed concurrently.

Effect of Acetylation on the Fluorescence Characteristics and Intensities of the Fluorophors derived from the Amines

Acetylation changed the wavelengths for maximum

fluorescence from adrenaline and dopa using the ethylenediamine method of assay but had no marked effect on the fluorescence characteristics of the other amines (Table 8 ). With the exception of metanephrine assayed by the ethylenediamine method, acetylation did not affect the sensitivity of assay of adrenaline and metanephrine. However, the sensitivity of the assay of noradrenaline and normetanephrine was decreased and that of dopamine and methoxydopamine increased, by acetylation. A measure of the fluorescence yields from each unacetylated and acetylated amine, by the method chosen for the assay of the acetylated derivative, is afforded by the steepness of their respective calibration curves (Figures 12, 13, 14 ). These curves demonstrate that the increase in the sensitivity of assay of dopamine and methoxydopamine by acetylation was due to an increase in the fluorescence yields from the amines and the decrease in the sensitivity of assay of noradrenaline after acetylation to a decrease in the fluorescence yield from noradrenaline.

The sensitivity of assay of an acetylated amine by a given technique, like the sensitivity of assay of an unacetylated amine (p. 80 ), varied on different occasions (Table 8 ). This variation was due to independent variations in the intensity of fluorescence obtained from a constant amount of amine and in the

size of the blank. In the case of the ethylenediamine assays, any increase in the blank could often be traced to ethylenediamine which had been in use for longer than the recommended two weeks (p. 82 ). The relative contribution of these two factors to the variation in the sensitivity of the estimation of a given amine depended on the amine being assayed and on the method of assay used i.e. whether it was the ethylenediamine or the trihydroxyindole method.

#### Comment on the Assay of Acetyl-normetanephine

When first examined in pure solution, the sensitivity of this assay was 45  $\mu\text{g}$ . Apparently coincident with a move to a new laboratory, the sensitivity fell to 100 - 200  $\mu\text{g}$ , without any significant alteration in the reagent blank fluorescence. It seemed possible that this reduction in fluorescence yield might have resulted from the change in the water supply used to make up the reagents, the original supply containing material which catalysed the fluorophor production. Definitive experiments however, have not given any evidence of this. It may be that such a catalyst was present in the reagents employed originally. Further investigations, particularly on the effect of copper, which has been reported (Häggendal, 1963) to accelerate the oxidation

of adrenaline and noradrenaline to their chrome derivatives, are proceeding.

Quantification of the Acetylation Procedure  
in Pure Solution

Welsh (1952a) found that 3.3 g of adrenaline could be quantitatively converted to triacetyl-adrenaline. Neither he nor Goldstein and co-workers (1959a and 1959b), however, ascertained if acetylation of adrenaline, noradrenaline, dopamine, metanephrine, normetanephrine, methoxydopamine and dopa was quantitative at microgram or millimicrogram levels i.e. at the amounts liable to be found in extracts of brain tissue. Two types of experiments were designed to investigate this question. In the first series of experiments (i) the degree of acetylation and degree of extraction of the acetylated compound into an organic solvent were looked at. In the second series of experiments (ii) the relative fluorescence from acetylated amines and dopa which had been acetylated in milligram and millimicrogram amounts was compared with the relative fluorescence obtained after acetylation of the same amines and dopa, in microgram quantities.

(i) 20  $\mu$ g amounts of the separate amines and dopa were acetylated and extracted into dichloromethane (p. 98) or ethyl acetate (p. 118). The aqueous solutions remaining after extraction of the acetylated amines into dichloromethane were acidified to pH about 3.5 with conc. hydrochloric acid. The aqueous solution remaining after extraction of acetyl-dopa, was adjusted from pH about 1, to pH about 3.5, using drops of 5N potassium carbonate.

The aqueous solutions from metanephrine, nor-metanephrine and methoxydopamine were divided into 4 portions. The acetylated methoxyamines were estimated in two of these portions (p. 117, 114, 110) and the unacetylated amines were estimated in the other two portions, using the same methods as were used to measure the acetylated derivatives, but without the initial hydrolysis step. To one portion from each pair was added an internal standard, to check for quenching of the fluorescence by the aqueous solution. Aliquots of the aqueous solutions remaining from the acetylation of adrenaline, noradrenaline, dopamine and dopa were assayed by the ethylenediamine method used to estimate their acetylated derivatives (p. 108, 119). Both unacetylated and acetylated amines are estimated by this technique but since the wavelength characteristics of the fluorophors from acetyl-adrenaline and

acetyl-dopa are quite different from those of the fluorophors from adrenaline and dopa it was possible to determine from the emission spectra, if any fluorescence from the aqueous solutions was derived from the acetylated or unacetylated form of adrenaline and dopa. In the cases of noradrenaline and dopamine, no such distinction is possible, since the acetylated forms of these amines, fluoresce maximally at the same wavelengths as the unacetylated amines (p.131 ). However, the observed fluorescence was no greater than the fluorescence from the corresponding blank, showing that no detectable acetylated or unacetylated noradrenaline or dopamine had been left in the aqueous phase (Table 9 ).

(ii) 100  $\mu$ g to 500  $\mu$ g amounts of the amines and dopa were acetylated and extracted into dichloromethane (p. 98 ) or ethyl acetate (p.119 ). After evaporating the solvent extracts to dryness, the residues were taken up in 3 - 4 ml water and the concentration of the appropriate acetyl derivative estimated, using the methods given on p. 108-119 and standard solutions prepared as on p. 107 .

15 mg adrenaline, noradrenaline and dopamine were acetylated as described for 'preparation of markers' (p. 107 ), to give a solution containing the equivalent of 10 mg/ml unacetylated substance in 50% (v/v)



TABLE 9

%Acetylation and % Extraction into Dichloromethane or Ethyl Acetate of duplicate Amounts of 20  $\mu$ g of the Amines and Dopa (as judged by the amount of unacetylated or acetylated compound, respectively, left in the aqueous).

Compound	% Acetylation	% Extraction into dichloromethane or ethyl acetate	% Acetylation and extraction into dichloromethane or ethyl acetate
Adrenaline (1)	< 99.4	< 99.3	} < 99.45 x < 99.4 = < 99
(2)	< 99.5	99.5	
Noradrenaline (1)	< 97.4	< 96.5	< 97.4 x < 96.4 = < 94
Dopamine (1)	< 96.5	< 99.5	} < 97.7 x < 99.55 = < 97
(2)	< 98.9	< 99.6	
Metanephrine (1)	< 98.5	< 97.9	} < 98.75 x < 97.2 = < 96
(2)	< 99.0	< 96.6	
Normetanephrine (1)	99.8	Not done	
(2)	< 99.4	Not done	
Methoxydopamine (1)	< 96.6	< 98.8	} < 98.6 x < 98.7 = < 96
(2)	< 97.1	< 98.6	
Dopa (1)	< 99.6	< 98.7	< 99.6 x < 98.7 = < 98

$$\% \text{ acetylation} = \frac{20 - (\text{unacetylated amine remaining in aq. in } \mu\text{g})}{20} \times \frac{100}{1}$$

$$\% \text{ extraction into dichloro-} = \frac{20 - (\text{acetylated amine remaining in aq. in } \mu\text{g})}{20} \times \frac{100}{1}$$

methane

TABLE 10

Acetylation of Millimicrogram and Milligram  
Amounts of the Amines and Dopa

[Recoveries calculated using acetyl standards  
equivalent to 2 µg amine or dopa/ml.]

Amine or dopa	Amount for acetylation	Amount estimated in acetyl- ated fraction
Adrenaline	100 µg 200 µg 15 mg	98 µg 224 µg 14.78 mg
Noradrenaline	100 µg 200 µg 15 mg	116 µg 190 µg 15.8 mg
Dopamine	250 µg 500 µg 15 mg	256 µg 432 µg 15.6 mg
Metanephrine	100 µg 200 µg	148 µg 238 µg
Normetanephrine	500 µg	393 µg
Methoxydopamine	250 µg 500 µg	268 µg 502 µg
Dopa	400 µg	440 µg

methanol. This solution was diluted to give the equivalent of 2  $\mu\text{g}/\text{ml}$  unacetylated substance and 0.1 ml and 0.05 ml portions assayed for the acetylated amine, using the appropriate 2  $\mu\text{g}/\text{ml}$  acetyl standard (p. 107) prepared by acetylation of 20  $\mu\text{g}$  of the amine.

The results of these experiments are given in Tables 9 (i) and 10 (ii). Table 9 shows that there was no detectable unacetylated amine or dopa left in the aqueous phase after acetylation and extraction of 20  $\mu\text{g}$  of each amine or dopa. The amines and dopa must therefore either have been destroyed or converted to their acetyl derivatives. Detectable amounts of the acetylated derivatives were not present in these same samples, implying total removal of the acetylated compounds into the organic solvents. Destruction of the amines or dopa by the acetylation process, if it occurs, must be negligible, since millimicrogram amounts of the amines and dopa could be quantitatively recovered from the organic phase which would contain the acetyl derivatives (Table 10). It would appear from the results of these control experiments that the method of acetylation employed produces quantitative acetylation of the amines and of dopa no matter whether these are present in milligram or millimicrogram amounts in the solution.

Chromatography of the Acetylated Amines

Separation of the six acetylated amines using the maximum distance of travel, i.e. with acetyl-methoxy-dopamine, the compound with the greatest  $R_f$ , located at the foot of a 46 cm long chromatogram, was possible with the slow solvent flow on a Whatman no. 20 paper, but not with the faster solvent flow on a Whatman no. 1 paper. The band widths obtained with the no. 1 paper were much greater than with the no. 20. Development of the chromatogram at  $37^{\circ}\text{C}$ , as recommended by Goldstein and co-workers (1959b) was not found practicable, because of the difficulty in maintaining an even temperature within the chromatography tank. Any temperature gradients across the tank resulted in differences in the distances travelled by a given acetylated amine on chromatogram strips at different positions in the tank (p. 104) so that the marker strips did not give a true indication of the positions of the separated amines on the sample chromatograms. A trial run in which a chromatogram was run at  $4^{\circ}\text{C}$  indicated that there were no problems with temperature gradients under these conditions but there were possibly poorer separations of the methoxy- from the catechol-amines.

Experience has shown that, for good separation, the filter paper curtain carrying the aqueous phase must be thoroughly wet before introducing the chromatogram.

The tanks were used once a week for 3 weeks after preparation and kept well sealed all this time. Organic and aqueous phases were replenished as required. Repeated use of the prepared tanks in this way appeared not to affect adversely the separations. Bush (1952) who introduced toluene:ethyl acetate:methanol:water = 10:1:5:5 as a solvent system for the separation of steroids, found that replenishment of the solvents as required, without renewing the whole set-up before each chromatographic run, gave satisfactory chromatograms. Even with all these precautions, the extent of separation of acetyl-noradrenaline from acetyl-normetanephine was not as great as would be desired (Figure 15). It is possible to increase this separation by continuing the chromatography for these two amines after removal of that portion of the chromatogram carrying the other four acetylated amines but this modification was not incorporated into the routine methodology because of the extra time required for the further re-equilibration and continued development of the chromatogram.

An amount of extract equivalent to 1.25 g rat brain applied to the origin of a chromatogram did not affect the distances of flow of marker amounts of the acetylated amines added to the brain extract before application to the chromatogram. In order to check that acetylation of the much smaller amounts of amine

Figure 15

Chromatogram of Acetylated Amines after  
 Development with the Organic Phase of Toluene:  
 Ethyl Acetate: Methanol: Water = 10: 1: 5: 5.  
 Descending chromatography for 11 hrs. at 20 - 21°C.  
 Distance (in cm.) of acetylated amines from the origin.

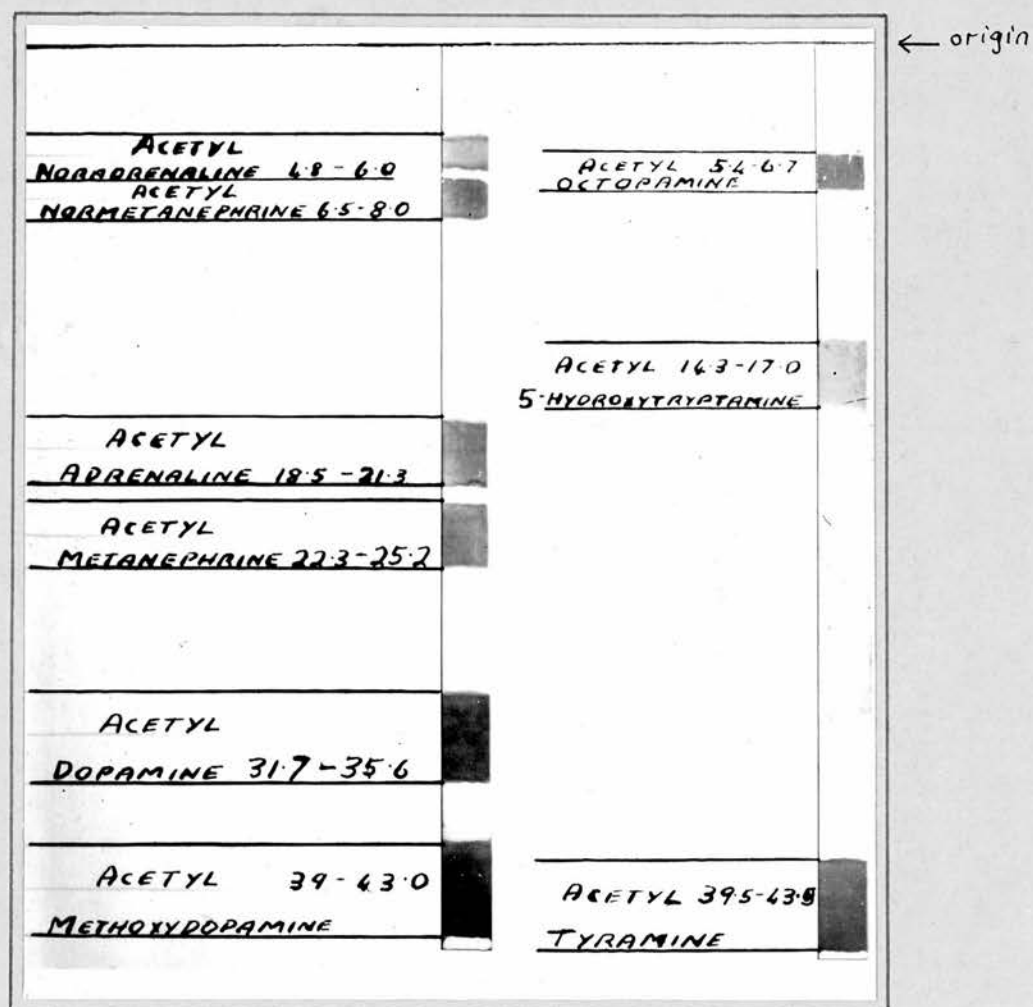
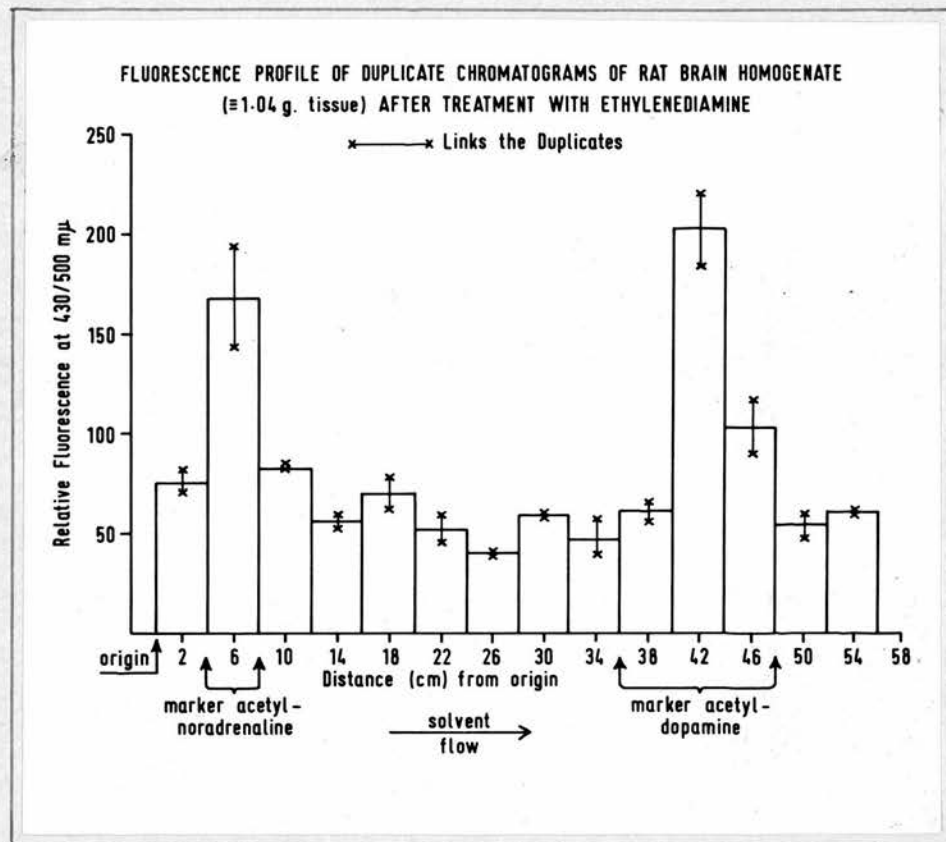




Figure 16



present in the brain extract produced acetylated derivatives with the same Rf as acetylated markers, chromatograms were run with duplicate portions of rat brain extract, each equivalent to 1.04 g tissue, after extraction and acetylation as on p.94-98. These 56 cm long chromatograms were accommodated in an extra-long chromatography tank, the use of which was subsequently abandoned because of difficulties with its sealing and equilibration. After development, the chromatograms were cut into 4 cm sections starting at a line 2 cm from the origin, each section was eluted in 3 ml water and a 2 ml portion put through the ethylenediamine assay procedure for the catecholamines (p.108 ). The fluorescence profile thus obtained (Figure 16 ) demonstrates that noradrenaline and dopamine endogenous in rat brain, after acetylation, showed the same distribution along a chromatogram as their corresponding aqueous visualised markers. Aqueous markers were therefore used routinely (p.107).

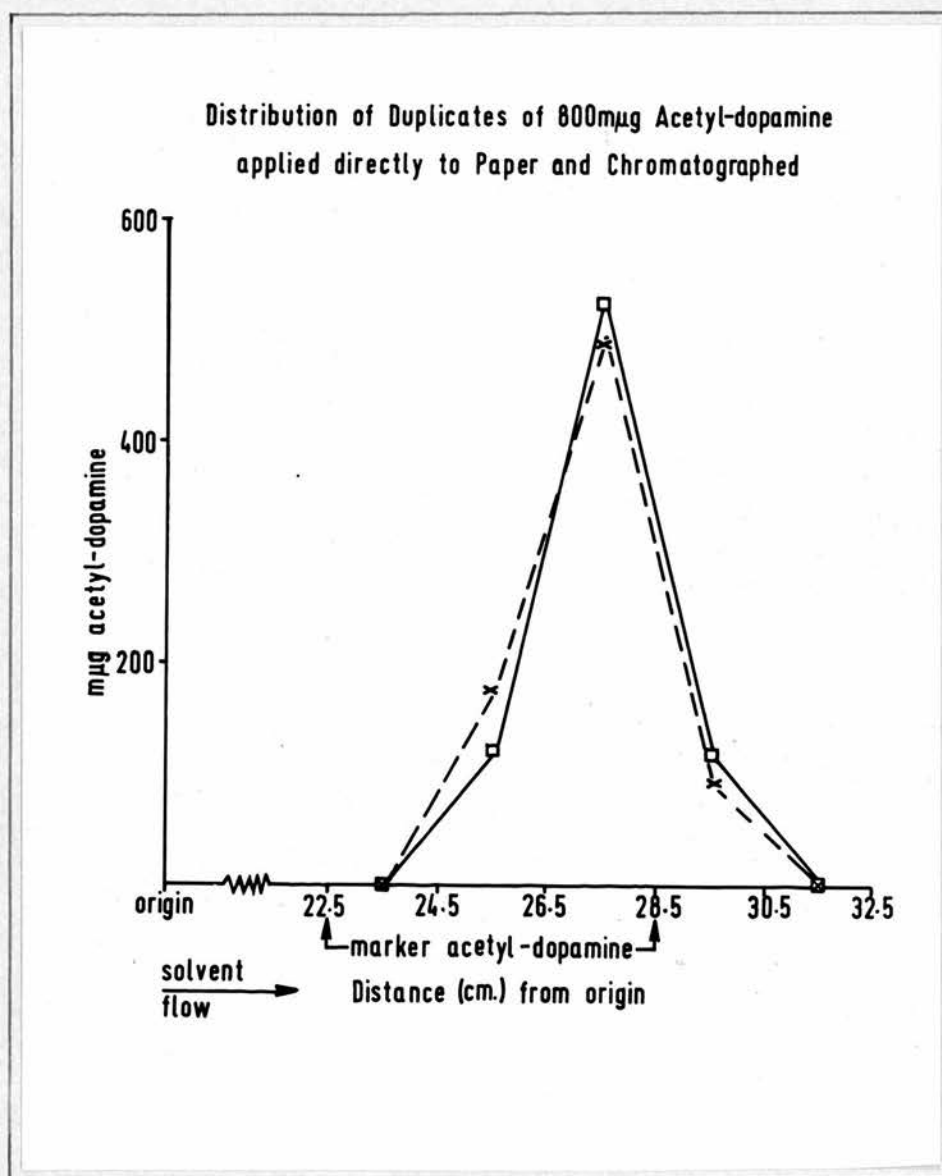
A photograph of a typical chromatogram of the acetylated catechol- and methoxy-amines is shown in Figure 15 . Also shown in this Figure are the distances of flow of acetyl-octopamine, acetyl-5-hydroxy-tryptamine and acetyl-tyramine. These acetylated amines were prepared in exactly the same way as the acetylated catecholamines (p.98 ). They were

investigated because all three are possible constituents of a dichloromethane extract from an acetylated brain extract. 5-Hydroxytryptamine in high concentration might interfere with the estimation of noradrenaline (p. 35). Such a contingency is avoided by the fact that by chromatography in toluene:ethyl acetate:methanol:water = 10:1:5:5, acetyl-5-hydroxytryptamine is separated from the acetylated derivatives of the catechol- and methoxy-amines (Figure 15). Octopamine and tyramine have not been detected in normal brain but have been found in the brains of animals pre-treated with monoamine oxidase inhibitors (Kakimoto and Armstrong, 1962). The acetylated derivatives of homovanillic acid, vanillinmandelic acid, dopa and tyrosine and the unacetylated amines did not move from the origin in this system.

The following colours were obtained on visualising with the alkaline diazotised p-nitroaniline reagent: acetyl-noradrenaline and acetyl-adrenaline, deep blue; acetyl-normetanephine and acetyl-metanephine, deep purple; acetyl-dopamine, acetyl-methoxydopamine and acetyl-tyramine, grey-blue; acetyl-5-hydroxytryptamine, light orange and acetyl-octopamine, rose-pink.

#### The Recoveries of Acetylated Amines from the Chromatographic Procedure

Two experiments were carried out to check

Figure 17

quantification of the chromatography, (i) in the absence of, and (ii) in the presence of, brain tissue. (i) Duplicate samples of 400  $\mu$ g acetyl-noradrenaline and 800  $\mu$ g acetyl-dopamine were applied directly to chromatogram strips which were developed (p. 101) along with appropriate marker chromatograms. Each chromatogram was cut into 2 - 3 cm long pieces over the area corresponding to marker acetyl-noradrenaline or acetyl-dopamine and each piece was eluted in 3 ml water. Acetyl-noradrenaline or acetyl-dopamine (p. 108) was estimated in the appropriate eluates.

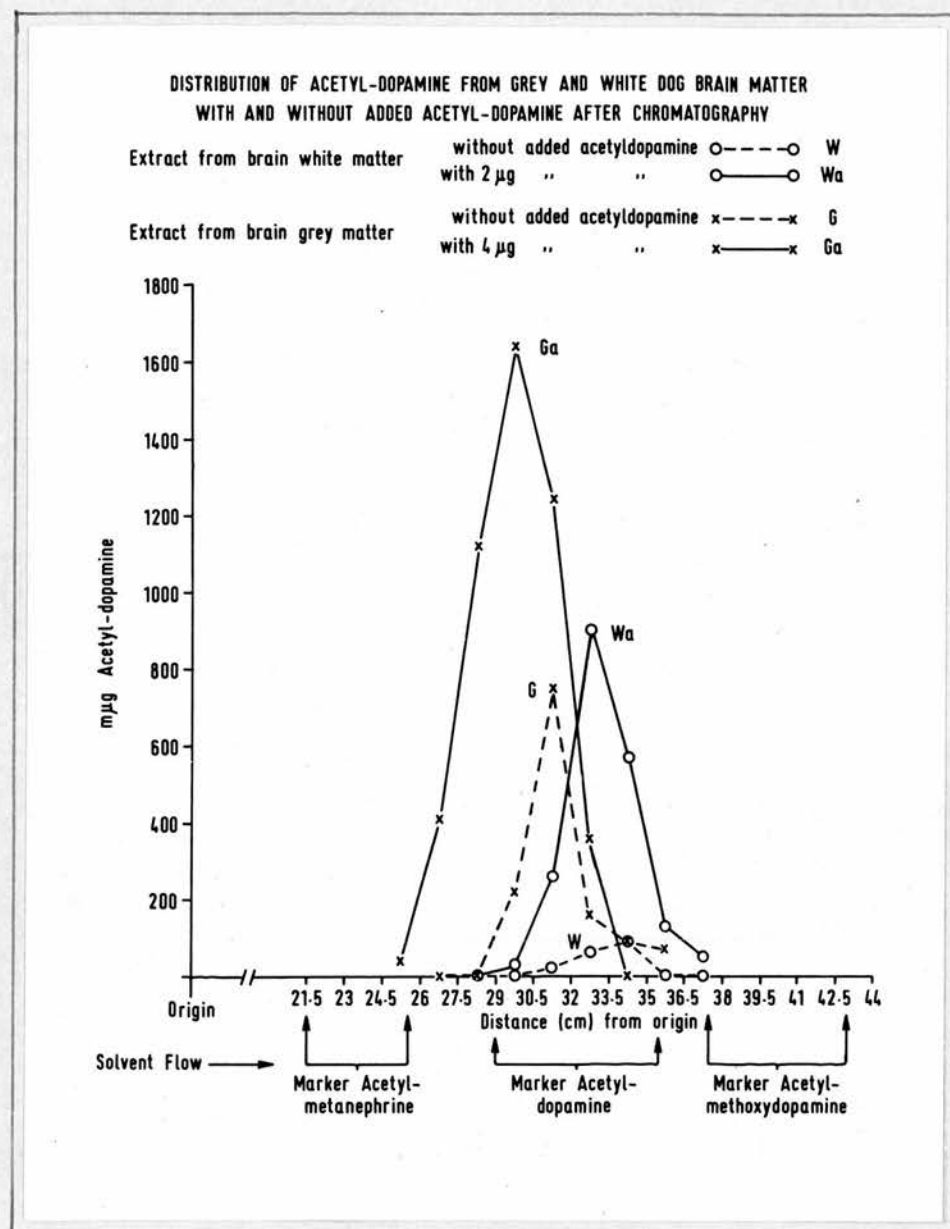
84% and 105% of the applied acetyl-noradrenaline and 94% and 95% of the applied acetyl-dopamine were recovered from the chromatograms. Acetyl-noradrenaline was found only in the acetyl-noradrenaline marker position; none was found in the neighbouring acetyl-normetanephine position. No partially acetylated dopamine was detected in the 10 cm of the acetyl-dopamine chromatograms including and extending from, the origin. Acetyl-dopamine appeared to be normally distributed over a 6 cm wide band corresponding to the marker position with a 2 cm extension - i.e. part of the 800  $\mu$ g acetyl-dopamine travelled 2 - 3 cm farther than was indicated by the colour reaction shown by the marker (Figure 17). The acetyl-dopamine found in this position was 12% and 16% of the total

acetyl-dopamine recovered in the two samples.

(ii) Application of this acetylation technique to the estimation of catecholamines and their metabolites in discrete areas of dog brain was anticipated. Since different areas of brain contain differing proportions of grey and white matter, it was considered advisable to ascertain if these two types of tissue differed in their effect on the disposition of the acetylated amines along the chromatogram. Acetyl-dopamine was chosen as the test amine because it had a relatively high  $R_f$  value and therefore any influence of tissue constituents might be expected to be the more evident.

That part of a dog's brain remaining after removal of the caudate nuclei, thalami, hypothalamus, midbrain and globus pallidus was dissected into two portions corresponding to the grey and the white matter, respectively. About 2 g of grey and about 2 g of white matter were extracted and acetylated and each divided into two aliquots. Acetyl-dopamine (4  $\mu$ g) was added to one aliquot of the extract from the grey matter and acetyl-dopamine (2  $\mu$ g) to one aliquot of the extract from the white matter, just before application to the chromatograms. After development, the first 6 cm of each chromatogram was divided into 4 x 1.5 cm sections which were eluted in 3 ml water.



Figure 18

These eluates were examined for any partially acetylated dopamine which, according to Lavery and Sharman (1965), could be found in this region of the chromatogram. The area of each chromatogram corresponding to marker acetyl-dopamine, as well as portions in front of and behind this area, was divided into 1.5 cm pieces and each piece eluted in 3 ml water. The eluates were assayed for acetyl-dopamine as described on p. 108.

88% of the applied acetyl-dopamine was recovered from both grey and white brain matter samples. Only in the recovery sample from the white matter was there any trace of material producing a fluorophor with the characteristic maxima, at 430/520 m $\mu$ , of that derived from acetyl-dopamine, at or within 6 cm of the origin. This 'acetyl-dopamine' was found at the origin of the chromatogram and amounted to 4.5% of the acetyl-dopamine recovered from the acetyl-dopamine position. The band spread of the marker acetyl-dopamine on a developed chromatogram shown in Figure 18 is 7 cm and includes the extremes of the 3 - 4 cm wide bands obtained from each of the three marker chromatograms. This slight difference in the R<sub>f</sub> of acetyl-dopamine on different chromatogram strips may be due to a temperature gradient across the tank (p. 139). Acetyl-dopamine recovered from the sample chromatograms showed an



approximately normal distribution, except for a tendency to 'tail' at the solvent front ends of the distributions (Figure 18 ). This 'tailing' was proportionately greater in chromatograms carrying 1 - 2  $\mu$ g amounts of the amine ((G)) and (Wa) in Figure 18 ) than in a chromatogram carrying 4.8  $\mu$ g acetyl-dopamine ((Ga) in Figure 18 ).

The positions of the acetyl-dopamine on the chromatograms from the white matter sample ((W)) and from its duplicate containing added acetyl-dopamine ((Wa)) were, with the exception of some 'tailing' in the case of (Wa), the same as the position of the marker acetyl-dopamine. The slight difference between the positions of the acetyl-dopamine distributions from these two samples could be entirely attributed to the slight difference, probably caused by a temperature gradient within the tank, in the Rf of acetyl-dopamine as determined from marker chromatograms located at different positions in the tank. There was thus no evidence that extracts of white matter affected the disposition of acetyl-dopamine on the developed chromatogram. The difference between the positions of acetyl-dopamine from the grey matter extract ((G)) and from its duplicate containing added acetyl-dopamine ((Ga)), could not be explained entirely by a temperature gradient across the tank and must be

due in part to a retarding effect of materials present in the grey matter extract, on exogenous acetyl-dopamine. This retardation, combined with the large amount of acetyl-dopamine (4.8  $\mu$ g) present in (Ga), resulted in a loss of 1% of the acetyl-dopamine into the acetyl-metanephrine position.

The band spread of acetyl-dopamine on the developed chromatograms appeared to be a function of the amount of acetyl-dopamine present e.g. in sample (Ga) containing 4.8  $\mu$ g the band spread was 9 cm, whereas in sample (W) containing 0.18  $\mu$ g the band spread was reduced to 4.5 cm.

Recovery of Mixtures of the Amines and Dopa  
through the Acetylation and Chromatography

Two experiments, each containing four samples, were carried out. In the first experiment only the recoveries of the amines were investigated but in the second experiment dopa was included. The amine mixtures were acetylated in 5 ml 0.005N hydrochloric acid containing also, in experiment (2), 600  $\mu$ g ascorbic acid and 2.2 mg disodium edetate, added to protect the amines against oxidation and attack by metal ions (p. 9 ). In each experiment, two samples had five times as much noradrenaline as dopamine and two samples five times as much dopamine as noradrenaline.

TABLE 11

% Recoveries of mixtures of the amines and dopa in pure solution through the acetylation and chromatographic procedures.

Experiment	Sample	Dopa	Noradren- aline	Normeta- nephrine	Adrena- line	Metane- phrine	Dopamine	Methoxy- dopamine
(1)	A		89	75	23	92	93	99
	B	Not	83	83	28	102	106	89
	C	done	64	34	9	48	23	47
	D		100	116	25	90	74	101
(2)	A	75	87	25	72	92	106	99
	B	100	83	19	70	100	106	83
	C	62	88	0	85	75	100	94
	D	70	62	0	68	65	92	94

	Samples	
	A and B	Cand D
Noradrenaline	1.0 µg	0.2 µg
Adrenaline, Metanephrine, Normetanephrine	0.4 µg	0.2 µg
Dopamine	0.2 µg	1.0 µg
Methoxydopamine, Dopa	0.2 µg	0.4 µg

Acetylation, chromatography and assay were as described under 'Methods'.

The results are given in Table II . All samples fluoresced maximally at wavelengths characteristic of the fluorophor from the substance for which they were being assayed, with the exception of solutions containing acetyl-normetanephrine and solutions containing 0.2 ug or less of acetyl-dopa. The results demonstrate that in pure solution, the components of a mixture of adrenaline, noradrenaline, dopamine, metanephrine, normetanephrine, methoxydopamine and dopa can be quantitatively separated and estimated as their acetyl derivatives.

The low recoveries from C in experiment (1) appeared to result from de-acetylation of the acetylated amines, possibly by traces of acid from the walls of the round-bottomed flasks in which the dichloromethane extracts were evaporated to dryness (p. 99 ), caused by insufficient rinsing of the flask after it had been cleaned in chromic acid. Elution of a 3 cm section from and including the origin of chromatogram C and treatment of this eluate with ethylenediamine produced a fluorescence which corresponded qualitatively and approximately quantitatively with the acetyl-dopamine unaccounted for in the acetyl-dopamine eluate from this chromatogram. Similar treatment of eluates

from the same position on chromatograms A, B and C in experiment (1) yielded fluorescences no greater than that from the corresponding paper blank. Some dopamine-like fluorescence (i.e. a fluorescence showing maximal intensity at 430/520 m $\mu$ ) was also detected in the eluate from a section of chromatogram C located at 2.0 to 3.5 cm from the origin. This situation was strongly reminiscent of the retention at the origin of the paper chromatogram, of breakdown products, presumably the partially hydrolysed derivatives, on developing the acetylated amines on acidic chromatography paper (p. 112). It can be inferred that exposure of the acetylated amines to acidic conditions before or during chromatography interferes with their quantitative recovery from the chromatogram by causing an uncontrolled partial de-acetylation, followed by separation of the partially acetylated amines from the fully acetylated amines, by the chromatographic procedure.

The most probable explanation for the low recoveries of adrenaline in experiment (1) is a 4-fold error in the preparation of the adrenaline dilution. The low recoveries for acetyl-normetanephrine in experiment (2) can be explained by failure of the assay method, which even in experiment (1) was not very satisfactory in that the sensitivity of assay was only 200 m $\mu$ g and no characteristic fluorescence maxima could be obtained

TABLE 12

Catecholamines, their amine metabolites and dopa in rat brain as  $\mu\text{g/g}$  tissue (uncorrected for recoveries). Three rat brains (minus cerebellums) pooled per experiment. Extracted using 0.4N P.C.A.

Experiment	Samples	Dopa 0.4 $\mu\text{g}$ or 4 $\mu\text{g}$	Noradren- aline 0.2 $\mu\text{g}$	Normeta- nephrine 0.2 $\mu\text{g}$	Adrena- line 0.2 $\mu\text{g}$	Metane- phrine 0.2 $\mu\text{g}$	Dopamine 0.2 $\mu\text{g}$ or 0.5 $\mu\text{g}$	Methoxy- dopamine 0.2 $\mu\text{g}$ or 0.5 $\mu\text{g}$
(1)	Recoveries							
	Duplicates	-	0.20	<0.05	<0.02	<0.02	0.79	0.05
	% Recovery	34	0.24	<0.05	<0.02	<0.02	0.74	0.05
			0	0	32	41	0	0
(2)	Recoveries							
	Duplicates	-	0.14	<0.05	<0.02	<0.02	0.56	<0.05
	% Recovery	48	0.19	0.04	<0.02	<0.02	0.90	<0.05
			0	0	18	51	72	0
(3)	Recoveries							
	Duplicates	-	0.17	<0.05	<0.02	<0.02	0.77	0.07
	% Recovery	50	0.14	<0.05	<0.02	<0.02	0.70	0.10
			32	18	53	59	69	0
(4)	Recoveries							
	Duplicates	-	0.13	<0.05	<0.02	<0.02	0.64	<0.05
	% Recovery	54	0.22	<0.05	<0.02	<0.02	0.75	0.10
			0	40	56	48	123	0
(5)	Recoveries							
	Duplicates	-	0.13	<0.05	<0.02	<0.02	0.48	<0.05
	% Recovery	0	0.13	0.05	<0.02	<0.02	0.62	<0.05
			lost	41	34	35	56	42
	Mean ± S.D.		0.17 0.03	0.02	0	0	0.70 0.08	0.06

S.D. - standard deviation calculated from means of duplicates.

- in dopa assays means that, judging from the emission spectrum, no dopa was present.



with this amount of standard acetyl-normetanephrine. The reason for this reduced sensitivity is discussed on p. 132 .

The sensitivity of assay of acetyl-dopa after extraction into ethyl acetate (p. 119 ) was 200  $\mu\text{g}$  i.e. five times less than the sensitivity of acetyl-dopa estimation in pure solution, because of the relatively high ethyl acetate extract blank and of the slight (15%) quenching of the fluorescence produced from 100  $\mu\text{g}$  acetyl-dopa by the ethyl acetate extracts. The high extract blank was found to arise from the residue from evaporation of 20 - 30 ml ethyl acetate to dryness (p. 119 ).

#### RESULTS OF ESTIMATIONS OF CATECHOLAMINES, THEIR AMINE METABOLITES AND DOPA IN RAT BRAIN

The results of five experiments, using homogenates, each prepared from three pooled rat brains (p. 94 ) are given in Table 12 . Each estimate was obtained from a chromatogram to which an extract equivalent to 1.1 g rat brain had been applied.

The estimates for noradrenaline in rat brain varied from 0.13 to 0.24  $\mu\text{g/g}$  brain tissue with a mean of 0.17  $\mu\text{g/g}$  and those for dopamine from 0.48 to 0.79  $\mu\text{g/g}$  with a mean of 0.70  $\mu\text{g/g}$ . Adrenaline and metanephrine concentrations were below 0.02  $\mu\text{g/g}$ , the



TABLE 13

## Sensitivity of Estimation of Amines and Dopa in Rat Brain Extracts

Level of detection is calculated on the basis of an average single sample wt. of 1.1 g brain and using half the assay sensitivity as equal to the level of detection

Acetylated Derivatives of:	Sensitivity of Assay ( $\mu\text{g}$ )		Level of Detection of Unacetylated Amines or Dopa as $\mu\text{g/g}$ Rat Brain
	Pure Soln.	Brain Paper Eluates	
Dopa	0.04	0.4	0.27
Noradrenaline	0.03	0.06	0.04
Normetanephhrine	0.045	0.06	0.05
Adrenaline	0.02	0.03	0.02
Metanephhrine	0.01	0.015	0.02
Dopamine	0.015	0.04	0.03
Methoxydopamine	0.02	0.06	0.05

limiting concentration for detection of these amines (Table 13 ). The fluorescences derived from the acetyl-dopamine and acetyl-noradrenaline fractions from the brain extracts were maximal at the wavelengths of maximal fluorescence of the acetyl-dopamine and acetyl-noradrenaline fluorophors, respectively. Unfortunately the value of this finding as an indication of the specificity of the acetyl-noradrenaline estimation is reduced by the fact that the fluorescence derived from the paper blank was maximal at the same wavelengths as that derived from acetyl-noradrenaline. Only two estimates were obtained for brain normetanephrine, both at the limit of detection by the method. The fluorescence intensity was too low to give rise to excitation and emission spectra showing the maxima characteristics of the normetanephrine fluorophor.

Five estimates, varying from 0.05 to 0.10  $\mu\text{g/g}$  were obtained for methoxydopamine in rat brain. The fluorescence from which these estimates were calculated, was maximal at wavelengths characteristic of the fluorophor derived from authentic acetyl-methoxydopamine. Eluates from the acetyl-methoxydopamine position of five other brain chromatograms contained no detectable acetyl-methoxydopamine, i.e. were derived from samples containing 0.05  $\mu\text{g}$  methoxydopamine/g brain tissue. The wavelengths of maximal fluorescence obtained from

these eluates were the same as those obtained from their paper blanks and were quite different from the characteristics of the acetyl-methoxydopamine fluorophor.

Dopa could not, strictly speaking, be estimated using the method in hand because there was no tissue blank for the acetyl-dopa assay. However, judging from the emission spectrum of the samples, there was no dopa in rat brain.

As already reported under each assay in the 'Methods' section, quenching of the fluorescence from each of the acetylated amines, by eluates from brain chromatograms, was not significant. The sensitivities of assay of the amines in the presence of eluates from rat brain chromatograms were less than the corresponding sensitivities in pure solution because the paper blanks were greater than the reagent blanks. Table 13 compares these sensitivities and lists the levels of detection of each compound per g rat brain tissue using the methods of analysis described on p. 108-119.

The reasonably consistent values for brain amines were accompanied by extremely variable and poor recoveries of added amines through the whole procedure. In the better recoveries each compound showed maximum fluorescence at the same wavelengths as its aqueous standard, with the exception of 0.4  $\mu$ g dopa. Increasing the amount of dopa to 4  $\mu$ g gave sufficient

acetyl-dopa in the recovery sample to produce a fluorescence peak at the wavelengths characteristic of acetyl-dopa i.e. at 430/540 mμ. The sensitivity of the acetyl-dopa assay was only 400 mμg because of the high extract blank (p. 156) and because of the 50% quenching, by the brain extracts, of the fluorescence from 100 - 200 mμg acetyl-dopa added as internal standard.

Two alternative methods of extraction of the brains were investigated in attempts to improve the recoveries. In the first method, the brains were homogenised in 2 - 3 vol. 0.1N hydrochloric acid and protein precipitated by addition of sufficient 4N perchloric acid to produce a final concentration of 0.4N perchloric acid. This homogenate was then divided into 3 portions and known amounts of the amines and dopa added to one of these portions, as on p. 95 to determine the recovery of each substance through the procedure, which was, from then on, the same as that described in the 'Methods' section. In two out of three experiments using this technique ascorbic acid 500 μg/g brain tissue was present during the initial 0.1N hydrochloric acid homogenisation. A troublesome frothing occurred during homogenisation in 0.1N hydrochloric acid although never during homogenisation in 0.4N perchloric acid.

TABLE 14

Catecholamines, their amine metabolites and dopa in rat brain as  $\mu\text{g/g}$  tissue (uncorrected for recoveries). Three rat brains (minus cerebellums) pooled per experiment. Extracted using 0.1N  $\text{H}_2\text{SO}_4$  or 0.1N HCl + P.C.A. to 0.4N P.C.A.

Experiment	Recoveries	Dopa 0.4 $\mu\text{g}$	Noradren- aline 0.4 $\mu\text{g}$	Normeta- nephrine 0.4 $\mu\text{g}$ or 0.2 $\mu\text{g}$	Adrena- line 0.4 $\mu\text{g}$ or 0.2 $\mu\text{g}$	Metane- phrine 0.4 $\mu\text{g}$ or 0.2 $\mu\text{g}$	Dopamine 0.8 $\mu\text{g}$ or 0.4 $\mu\text{g}$	Methoxy- dopamine 0.4 $\mu\text{g}$ or 0.2 $\mu\text{g}$
(6) homo. in 0.1N HCl + P.C.A.	Duplicates % Recovery	- - 44	0.11 0.46 0	<0.05 0.16 75	0.02 0.02 24	<0.02 <0.02 150	0.24 0.44 20	0.08 0.19 57
(7) + a homo. in 0.1N HCl + P.C.A.	Duplicates % Recovery	- - 0	0.19 <0.04 39	0.06 0.04 51	0.02 <0.02 32	<0.02 <0.02 71	0.85 1.13 0	0.19 0.12 44
(8) + a homo. in 0.1N HCl + P.C.A.	Duplicates % Recovery	- - 0	0.05 0.08 98	<0.05 <0.05 225	<0.02 <0.02 26	<0.02 <0.02 60	0.06 0.13 58	lost 0.07 89
(9) homo. in 0.1N $\text{H}_2\text{SO}_4$	Duplicates % Recoveries	- - 84 44	0.22 0.65 8 39	0.07 0.14 0 0	<0.02 <0.02 36 32	<0.02 <0.02 48 34	1.03 1.22 116 90	0.09 0.10 0 0

'+' indicates 500  $\mu\text{g/g}$  ascorbic acid added during homogenisation in HCl.

'-' in dopa assays means that, judging from the emission spectrum, no dopa was present.

In the second method, the brains were homogenised in 4 vol. 0.1N sulphuric acid, the homogenate divided into 4 portions and known amounts of the amines and dopa added to 2 of these portions, to serve as recovery estimates. On centrifugation at 6000 g for 7 min. these homogenates yielded proteinaceous supernatants, which cleared somewhat after the next step, which was saturation with sodium chloride followed by extraction with ethyl acetate to remove the acid metabolites (p.97). Addition of drops of 2N hydrochloric acid as on p.97, previous to ethyl acetate extraction was unnecessary since the sulphuric acid supernatants were sufficiently acid (pH 1.5) to suppress ionisation of the carboxylic acids. After this extraction, the procedure was exactly the same as that described in the 'Methods' section.

The results of three experiments using homogenisation in 0.1N hydrochloric acid and of one experiment using homogenisation in 0.1N sulphuric acid are given in Table 14. The recoveries were better using an initial homogenisation in 0.1N hydrochloric acid, especially if ascorbic acid was added but the estimates for brain amines varied much more widely than when the tissue was homogenised directly in 0.4N perchloric acid. Despite the apparent poor protein precipitation with sulphuric acid, the amine values obtained using



this technique were quite reasonable, though perhaps rather high but the recoveries were poor.

Since it was the only one of the three tested methods of extraction which gave consistent figures for rat brain amines, homogenisation directly in perchloric acid was considered the best extraction method for studies on brain amines. A disturbing feature of the experimental results was the variable and sometimes marked, losses of the exogenous amines and dopa added to portions of the brain homogenates, and analysed in parallel with the brain samples. Investigations into the causes of these losses were continued, using the method of homogenising in perchloric acid, in the next series of experiments (Section II). In this series the recovery investigations were done at the same time as studies on the levels of catechol- and methoxy-amines in discrete areas of brains taken from control dogs and from dogs which had received various drugs. It was considered justifiable to use the perchloric acid homogenisation method for such studies, because using this method the estimates for the amines in rat brain without any correction based on the recoveries of exogenous amines, were consistent, despite highly variable recoveries.



## DISCUSSION

Acetylation and Estimation of the Acetylated Amines

If, as according to Welsh (1955), acetylation occurs of the phenolic hydroxyls and amino nitrogen, adrenaline, noradrenaline, dopamine and dopa should yield tri-acetyl derivatives and metanephrine, nor-metanephrine and methoxydopamine di-acetyl derivatives. Evidence in support of the tri-acetyl formula for acetyl-dopamine was obtained using chromatography and fluorescence reactions. If acetyl-dopamine was chromatographed in acid solution, as happened following the use of acid contaminated glass-ware (p. 154 ) or acid contaminated paper (p. 112 ), dopamine derivatives were recovered from three positions on the chromatogram, namely (i) the origin, (ii) about 1 cm behind the acetyl-noradrenaline position and (iii) in the usual acetyl-dopamine position (p. 154). The amine at (iii) was fully acetylated tri-acetyl-dopamine, while (i) and (ii) were presumably breakdown products resulting from partial hydrolysis of tri-acetyl-dopamine by acid. Since phenolic acetates are hydrolysed fairly readily (Welsh, 1955) it seems likely that (i) was N-acetyl-dopamine with no phenolic acetyl groups and (ii) was N-acetyl-dopamine with one phenolic acetyl group. Since hydrolysis of substituents on the catechol nucleus

is a necessary preliminary to coupling with ethylenediamine (p.22 ), the phenolic acetyl groups cannot contribute to the potentiation of fluorescence. This leaves the N-acetyl group. The foregoing evidence strongly suggests that the N-acetyl linkage resists hydrolysis in weak acid or strong alkali and is responsible for the potentiation of dopamine fluorescence on coupling with ethylenediamine.

In order to quantify the chromatography, it is essential to obtain complete acetylation of dopamine to its tri-acetyl derivative, since incomplete acetylation, like partial de-acetylation (p.154), could give rise to a mixture of the three acetylated derivatives which would separate on chromatography. Brief investigations have indicated that a similar situation holds for acetyl-noradrenaline and acetyl-adrenaline. Hence precautions, such as thorough mixing (p.98 ) and removal of traces of ethyl acetate (p.97 ), were taken to ensure complete acetylation of each amine to its di- or tri- acetyl derivative.

The yield of fluorescence from the interaction of acetyl-noradrenaline with ethylenediamine, unlike that from acetyl-dopamine, was found to be less than from the parent amine. The fluorescence yield from acetyl-adrenaline was about the same as that from adrenaline, although the fluorescence characteristics differed (Table 8).

The observed excitation and emission wavelengths for maximum fluorescence for the fluorophors from acetyl-dopamine, acetyl-noradrenaline and acetyl-adrenaline were the same as those reported by Lavery and Sharman (1965). These workers, however, found that acetylation increased the fluorescence yield from noradrenaline and adrenaline as well as from dopamine. Since the fluorescence yield from adrenaline was found to be very much higher than that reported by Lavery and Sharman, this absence of potentiation on acetylation did not make the sensitivity of acetyl-adrenaline assay any lower than that found by Lavery and Sharman. The sensitivity of acetyl-noradrenaline assay, however, was lower and no explanation could be found for this discrepancy. Control experiments showed that it was not due to the fact that Lavery and Sharman used 2N hydrochloric acid to prepare their ethylenediamine reagent, whereas we used 4M ammonium chloride.

The characteristics and intensity of the ethylenediamine-dopa fluorophor were changed by acetylation suggesting a different fluorophor which, since it could not be extracted into isobutanol (p.120), resembled the dopa fluorophor (p.24) in that it retained its hydroxyl group. Acetyl-methoxydopamine, after acid hydrolysis and periodate to remove the phenolic acetyl and methyl groups respectively, gave with ethylene-

diamine a fluorescence which was indistinguishable from that from acetyl-dopamine. The fluorophor, like that from acetyl-dopamine (p. 165) was probably derived from the condensation of ethylenediamine with N-acetyl-dopamine.

The fluorescence obtained from acetyl-noradrenaline and acetyl-normetanephine following acid hydrolysis and treatment with potassium ferricyanide had only half the intensity of the fluorescence from equimolar amounts of the parent amines. Since the phenolic acetyl groups must be removed to allow formation of the quinone necessary for fluorophor production (p. 22), the most likely explanation for this reduced fluorescence is that the N-acetyl group resists the acid hydrolysis and that the fluorophor from N-acetyl-noradrenaline has only half the fluorescence intensity of the fluorophor from noradrenaline. A similar type of incomplete hydrolysis could explain why acid hydrolysed acetyl-methoxydopamine, put through the trihydroxyindole reaction (p. 128) gave a fluorescence yield only one quarter that from methoxy-dopamine. Adrenaline, metanephine, acetyl-adrenaline and acetyl-metanephine differed from the foregoing compounds in that they gave qualitatively and quantitatively identical fluorescences, suggesting that both O and N acetyl groups were readily removable by hydrolysis and

that the same fluorophor was involved in each case.

#### Chromatography of the Acetylated Amines

Separation of the acetylated catechol- and methoxy- amines from one another could be achieved, if the precautions detailed on p. 103 were taken. The relative distribution of the acetylated amines on the developed chromatogram was approximately the same as that reported by Goldstein et al. (1959a), although direct comparison is not possible because these workers developed the chromatogram at 37°C. They make no reference to difficulties, such as were encountered in this work, in controlling the reproducibility of the chromatography. On some days the acetylated amines travelled farther than on other days, in spite of the fact that the time and temperature of development was the same and the time of equilibration was kept at 8 - 10 hours. Differing degrees of vapour saturation may account for these day-to-day variations. These variations however, did not matter unless the distance travelled by the amines was so much reduced as to eliminate separations between the methoxy- and corresponding catechol- amines. A more serious problem was that in spite of all precautions taken (p. 104) to prevent temperature gradients within the tank, marker amines tended to run at slightly different speeds on chromatograms located



at different positions in the developing tank. These differences were only of consequence with the fastest running amines, namely acetyl-dopamine and acetyl-methoxydopamine and allowance could be made for these differences by eluting from sample chromatograms segments of paper of greater depth than indicated by the position of the markers; this was possible because acetyl-dopamine and acetyl-methoxydopamine were well separated from one another and from their nearest neighbour, acetyl-metanephine. Recent investigations, which are to be followed up, indicate that chromatography at 4°C gives a consistent flow of the markers on chromatograms located at different positions in the tank.

Octopamine and tyramine may occur in measurable quantities in the brains of animals treated with monoamine oxidase inhibitors (Kakimoto and Armstrong, 1962). The acetyl derivatives of these amines have Rf values similar to those of acetyl-noradrenaline and acetyl-methoxydopamine, respectively (p.144). Their presence in chromatogram eluates would not interfere with the estimation of acetyl-noradrenaline or acetyl-methoxydopamine since neither acetyl-octopamine nor acetyl-tyramine yield fluorophors when subjected to the assay procedures for acetyl-noradrenaline or acetyl-methoxydopamine.

Estimation of Catechol- and Methoxy-Amines in Rat Brain

Very few methods have been reported in the literature whereby catechol amines and their metabolites may be estimated in the same tissue sample. Simultaneous estimations have on the whole been restricted to those compounds which can be separated by paper or ion exchange chromatography. A maximum of three amines can be quantitatively separated using ion exchange columns of reasonable length (p. 14 ) and various 2 or 3 amine combinations such as noradrenaline and dopamine (Baird and Lewis, 1964), noradrenaline, normetanephrine and dopamine (Häggendal, 1962) and noradrenaline, dopamine and methoxydopamine (Carlsson and Waldeck, 1964) have been assayed in brain extracts. Assay of the acid metabolites has invariably been carried out on separate aliquots from those used for the amine estimates. For example, no report of the assay of dopamine and its major metabolite, homovanillic acid, in the same aliquot of brain tissue could be found in the literature. Hagopian, Dorfman and Gut (1961) described a technique, very similar to the technique described in this thesis, whereby dopamine could be separated from homovanillic acid but they did not estimate the separated compounds. The amino acid precursor, dopa, which under appropriate pH conditions may be adsorbed, along with the catecholamines, onto a



strong cationic resin, can be estimated in the same sample as its amine derivative but again, owing to the limited resolution of this method of separation (p. 14 ), the number of amines measurable in the same aliquot as dopa is only one (Wiegand and Perry, 1961) or two (Glowinski, Iversen and Axelrod, 1966). An advantage of the technique reported in this thesis is that the amino acids, the acids and the amines are obtained in three separate fractions. Interference of the components of one fraction with estimation of components of another fraction is thus eliminated. No attempts were made to assay acetyl-tyrosine which would be present in the same fraction as acetyl-dopa. Tyrosine could thus be separated from its amine derivative, tyramine. (p. 2).

The only comparable technique of analysis of brain tissue reported in the literature is that described by Matsuoka (1964). He devised a method for separating noradrenaline, dopamine, normetanephrine, dihydroxymandelic acid and dihydroxyphenylacetic acid in rabbit brain by adsorption of the catechols onto alumina, followed by separation of the components of the eluted catechol, and of the non-adsorbed non-catechol, groups by ion exchange chromatography. The concentrations of dihydroxymandelic acid and dihydroxyphenylacetic acid in normal rabbit brain quoted by him are suspiciously

high, possibly due to elution of a fluorescence yielding material from the ion exchange resin, similar to the material Häggendal (1962a) found could be washed off Dowex 50 (p. 14). From this point of view, paper chromatography is probably preferable to ion exchange chromatography, although paper can give rise to high blanks (p. 12 and Sharman, 1963). Glowinski, Iversen and Axelrod (1966) and Glowinski and Iversen (1966), separated  $C^{14}$  or  $H^3$  labelled dopa, noradrenaline, dopamine, total methoxyamines, total methoxy deaminated metabolites and total deaminated metabolites from rat brain, after administration of  $C^{14}$  tyrosine or  $H^3$  dopa, respectively. These measurements are not affected by drawbacks, such as elution of fluorescence yielding material from the ion exchange resin, which are inherent in the assay of millimicrogram amounts of unlabelled substances. Since vanillinmandelic acid was not separated from homovanillic acid and normetanephrine was not separated from methoxydopamine, it is not possible to distinguish noradrenaline from dopamine metabolism using this technique.

The estimates for rat brain noradrenaline varied from 0.13 to 0.22 with a mean of  $0.17 \pm 0.03$  ( $n = 5$ )  $\mu\text{g/g}$ . Each of these estimates was the average of duplicate analyses. The standard deviation of the difference between the duplicates was 0.04  $\mu\text{g/g}$ .

The figures for rat brain noradrenaline in the literature may be divided into 2 groups, (i) a group of eight estimates ranging from 0.32 to 0.58 with a mean of 0.46  $\mu\text{g/g}$  and (ii) a group of three estimates of 0.15, 0.19 and 0.22 which mean at 0.20  $\mu\text{g/g}$  (Table 7, p. 83). This difference may be due to seasonal (Montagu, 1957) or strain (Anton and Sayre, 1964) variations in the rats. Neither of these reasons, however, could account for the fact that the noradrenaline estimates obtained using the acetylation technique (Table 12, p. 155) were less than the noradrenaline estimates obtained using the phenol:hydrochloric acid technique (Table 4, p. 67). A possible explanation is that the ethyl acetate extraction incorporated into the former, but not the latter, technique was responsible for loss of some of the brain noradrenaline. Extraction of some brain noradrenaline into ethyl acetate could be inferred from the results in sub-section 1 (p. 84) and was confirmed by further experiments using discrete areas of dog brain (p. 222).

In eight of the ten samples of rat brain analysed the amount of normetanephrine was below the limit of detection by the method, which was 0.05  $\mu\text{g/g}$ . The other two samples were estimated to contain 0.05 and 0.04  $\mu\text{g/g}$ . In essential agreement with these findings are those of Carlsson and Lindquist (1962) who quote a concentration of 0.03  $\mu\text{g}$  normetanephrine/g rat brain.

In none of the rat brain samples was the adrenaline concentration above the limit of detection, which was 0.02  $\mu\text{g/g}$ . This is less than the reported values, which range from 0.04 to 0.07 with a mean of 0.05  $\mu\text{g/g}$  with the exception of one very high estimate of 0.21  $\mu\text{g/g}$  (Table 7, p. 83). It is possible that these figures are over-estimates, since most of them were obtained by differential estimation of small amounts of nor-adrenaline (p. 2). No metanephrine has been found in rat brain, either in these experiments or in the literature.

The estimates for rat brain dopamine varied from 0.55 to 0.76 with a mean of  $0.70 \pm 0.08 \mu\text{g}$  ( $n = 5$ ). This agrees closely with the reported figures, which average at 0.65  $\mu\text{g/g}$  (Table 7, p. 83). Each of the present estimates was the mean of duplicate estimates. The standard deviation of the difference between the duplicates was 0.1  $\mu\text{g/g}$ .

Estimates for methoxydopamine of 0.05, 0.05 and 0.08  $\mu\text{g/g}$  were obtained in three out of five experiments. No figures for rat brain methoxydopamine with which to compare these estimates could be found in the literature.

The consistency of the estimates of the levels of the catechol- and methoxy-amines in rat brain demonstrates that the method is reproducible and that the poor and variable recoveries of the amines and

and dopa added to brain extracts do not reflect the recoveries of the endogenous amines. The losses of exogenous amines appeared to be occurring at some point in the extraction procedure since (i) the amines and dopa could be quantitatively recovered in pure solution through the acetylation and chromatographic procedures (p. 153) and (ii) the presence of a tissue extract did not interfere with quantitative chromatography of acetyl-dopamine (p. 146). Further discussion of these variable recoveries is continued in Section II.

This technique is unique in that the catecholamines and their metabolites are separated and estimated in the same brain sample. It is less wasteful of time and experimental animals, than techniques using less rigorous separation methods in which pooling of tissues from several animals is required in order to obtain measurable quantities of the substance being estimated, in the aliquots taken for assay of each substance or group of substances. Since the amines are separated from one another and from their amino acid precursors and acid metabolites prior to their estimation, interference of any of these compounds in the assay of one amine, is prevented.

This separation method would appear to have particular application to studies of catecholamine

metabolism using radioactively labelled precursors, since it is essential to have complete separation of the amines and their metabolites before they can be measured radioactively.

This technique has been applied to a study of the action of chlorpromazine on catecholamine metabolism in discrete areas of dog brain (Section II) and to studies on the interactions of the 5-hydroxyindole and catecholamine pathways in brain (Moir, 1967).

## SECTION II

Adrenaline, Noradrenaline, Dopamine, Metanephrine,  
Methoxydopamine, Homovanillic Acid and  
Dihydroxyphenylacetic Acid in discrete Areas  
of Dog Brain and the effect of  
Chlorpromazine (5 mg/kg and 15 mg/kg)  
thereon.



## INTRODUCTION

The presence of noradrenaline and adrenaline in brain was first shown by von Euler (1947). Since noradrenaline had, at that time, recently been demonstrated in extracts of peripheral sympathetic nerves (von Euler, 1947 and 1949) the noradrenaline found in brain was assumed to be related to the sympathetic vasomotor fibres. Vogt (1954) showed that this was not so and that areas of dog brain associated with the central representation of the sympathetic nervous system viz. the hypothalamus, midbrain, medial thalamic nuclei and floor of the fourth ventricle, contained five to ten times more noradrenaline than other areas. The conclusion drawn from these results, namely that noradrenaline may function as a neurotransmitter in the central nervous system has received support from recent microscopic and histochemical studies (Hillarp, Fuxe and Dahlström, 1966). Mägnert, Levi and de Lorenzo (1964) isolated, by gradient density centrifugation of rat brain homogenates, a fraction containing 'nerve-ending particles' which liberated, on ultrasonic rupture, vesicles containing 25% of the total brain noradrenaline. These vesicles were similar morphologically to vesicles seen at synaptic junctions in rat brain cortex by electron microscopy (de Robertis, de Lores Arnaiz and de Iraldi, 1962). Thus, although

the ultimate criterion for a neurotransmitter, that it should be liberated from the nerve ending by stimulation of the nerve axon, has not as yet been satisfied for noradrenaline in the brain, noradrenaline would appear to be morphologically disposed in the brain for such a liberation to be possible.

Synthesis of noradrenaline in the central nervous system most probably occurs along the same pathway as in the adrenal medulla (Figure 1, p. 3). The ability of the brain to synthesise dopamine and noradrenaline in vivo has been demonstrated by the isolation of  $C^{14}$ -dopamine and  $C^{14}$ -noradrenaline in guinea pig brain after intraperitoneal injection of  $C^{14}$ -tyrosine (Udenfriend and Zaltzman-Nirenberg, 1963) and by the isolation of  $H^3$ -dopamine and  $H^3$ -noradrenaline in rat brain after intra-ventricular administration of  $H^3$ -dopa (Glowinski and Iversen, 1966). Both tyrosine and dopa pass readily from the bloodstream into the brain (Chirigos, Greengard and Udenfriend, 1960; Rosell, Sedvall and Ullberg, 1963). The following enzymes, necessary for the synthesis of noradrenaline from tyrosine, have been demonstrated in brain tissue: tyrosine hydroxylase, which forms dopa from tyrosine (Nagatsu, Levitt and Udenfriend, 1964); dopa decarboxylase, which forms dopamine from dopa (Holtz, 1959) and dopamine- $\beta$ -oxidase, which forms noradrenaline from dopamine (Udenfriend and Creveling, 1959).

The intermediate compounds in the synthesis of noradrenaline from tyrosine are dopa and dopamine (Figure 1, p. 3). Dopa has never been detected in normal brains (Anton and Sayre, 1964) probably because it is very readily decarboxylated to dopamine (Bertler and Rosengren, 1959a). In 1959 Bertler and Rosengren showed the presence of dopamine, in concentrations similar to those of noradrenaline, in brains from several species. They also studied the distribution of dopamine in dog, cow, cat and sheep brain. Contrary to what might be expected if dopamine was present solely as a precursor of noradrenaline, the concentrations of dopamine in the caudate nucleus and lentiform nucleus of these four species were found to be about fifty and twenty times, respectively, the concentration of noradrenaline in these same areas of brain. Other basal ganglia in other species, such as monkey putamen (Poirier and Sourkes, 1965), human putamen (von Bernheimer and Hornykiewicz, 1965) and human substantia nigra (Hornykiewicz, 1963) also contain very much more dopamine than noradrenaline. Damage to the basal ganglia caused by lesions and disease, with the production of extra-pyramidal dysfunction in experimental animals (Poirier and Sourkes, 1965) and man (Ehringer and Hornykiewicz, 1960) has been shown to result in a reduction in the

dopamine content of the basal ganglia. Although dopamine-containing vesicles have not been isolated from brain, as have noradrenaline-containing vesicles (p. 176), dopamine has been detected from perfusates of cat caudate nucleus following electrical stimulation of the cortex and sub-cortex (McLennan, 1964). There is therefore some evidence that dopamine acts as a neurotransmitter in the basal ganglia. In other areas of brain, such as the hypothalamus and midbrain, in which the concentrations of dopamine are less than the concentrations of noradrenaline (Bertler and Rosengren, 1959), it is possible that dopamine functions solely as a precursor of the latter amine.

Noradrenaline and dopamine appear to be metabolised in the brain, as in the periphery, by monoamine oxidase and catechol-O-methyl-transferase. Both these enzymes are widely distributed in brain (Bogdanski, Weissbach and Udenfriend, 1957; Axelrod, Albers and Clemente, 1959). In a study of the effect of various drugs on the uptake and metabolism of  $H^3$ -noradrenaline introduced into the lateral ventricle of the rat, Glowinski and Axelrod (1965) concluded that in the brain, as in the periphery (Kopin, Hertting and Gordon, 1962), monoamine oxidase is primarily responsible for the metabolism of amines released intraneuronally and catechol-O-methyl-transferase for the metabolism of amines released extraneuronally. Glowinski and Axelrod (1965) and Glowinski,

Kopin and Axelrod (1965) also concluded that a major part of the  $H^3$ -noradrenaline injected into the lateral ventricle of the rat was taken up by the noradrenaline storage sites in the neurons. A similar mechanism for the removal of part of intravenously administered adrenaline and noradrenaline by the peripheral tissues of the mouse was previously demonstrated by Iversen and Whitby (1962) and Whitby, Axelrod and Weil-Malherbe (1961).

The O-methylated metabolites of noradrenaline and dopamine, normetanephrine and methoxydopamine, have been demonstrated in brain tissue (Carlsson and Lindquist, 1962; Carlsson and Waldeck, 1964). Dihydroxyphenylacetic acid and homovanillic acid, the acid metabolites formed from dopamine, have also been found to be present in brain (Rosengren, 1960; Sharman, 1963). Dihydroxymandelic acid and vanillin-mandelic acid, the theoretical acid metabolites from noradrenaline, have not been detected. This is probably because the methods of estimation are not sufficiently sensitive. It appears probable that the aldehydes formed by the action of monoamine oxidase on noradrenaline and normetanephrine are, in the main, reduced to the corresponding alcohols rather than oxidised to the acids. For example, Rutledge and Jonason (1967) found that on incubation with  $H^3$ -noradrenaline and  $H^3$ -dopamine, cortical slices from

the rabbit formed dihydroxyphenylglycol and 3-methoxy, 4-hydroxyphenylglycol from noradrenaline and dihydroxyphenylacetic acid and homovanillic acid from dopamine.

A method of studying the effect of a drug on brain catecholamines is to estimate the catecholamines, their precursors and metabolites, in brain, before and after administration of the drug. By comparing the estimates obtained from the 'control' and 'treated' animals, it should be possible to deduce whether the drug affects amine storage, synthesis or metabolism (Juorio and Vogt, 1965; Glowinski and Baldessarini, 1966; Lavery and Sharman, 1965). In this section of the thesis, an investigation of this nature was carried out into the action of chlorpromazine on catecholamine metabolism in dog brain, using the technique described in the preceding section (I).

The effect of chlorpromazine on the levels of homovanillic acid and dihydroxyphenylacetic acid in the lateral ventricular C.S.F. of the dog was shown,



in this department (Guldberg, 1967, p. 99), to be dependent on the dose, the duration of time between administration of drug and sampling of the C.S.F. and to some extent, the individual animal. In most dogs, 5 mg/kg intravenous chlorpromazine produced increases in the concentrations of homovanillic acid and dihydroxyphenylacetic acid which were maximal at 4 and 2 hr., respectively, after administration of the drug. Since the caudate nucleus forms a large part of the wall of the lateral ventricle and since high concentrations of homovanillic acid and dihydroxyphenylacetic acid have been found in this area in rabbits (Anden, Roos and Werdinius, 1964), it seemed probable that the increase in the concentrations of homovanillic acid and dihydroxyphenylacetic acid in the lateral ventricular C.S.F. was derived from an increase in the concentrations of these acids in the caudate nucleus. In order to test this hypothesis and to investigate the mechanism of this increase, the concentrations of dopamine and its acid and amine metabolites in the caudate nucleus of the dog 2 hr. after administration of chlorpromazine were determined. If the rise in dihydroxyphenylacetic acid in the ventricular C.S.F. following chlorpromazine was due to the metabolism by monoamine oxidase, of dopamine released from stores in the caudate nucleus, the time of maximal action



of chlorpromazine on the stores might be expected to be about the same as the time for the concentration of dihydroxyphenylacetic acid to reach its peak in the C.S.F. - i.e. two hr.. In the experiments the globus pallidus was analysed in addition to the caudate nucleus, in order to determine if chlorpromazine affected similarly the dopamine metabolism in the two different basal ganglia. The effect of chlorpromazine on catecholamine metabolism was also investigated in those areas of brain known to contain relatively high concentrations of noradrenaline namely the hypothalamus, midbrain and thalamus. Cortex was included as being representative of those parts of the brain containing low concentrations of catecholamines.

Similar experiments were also performed on dogs killed 2 hr. after a three-fold larger dose of chlorpromazine, namely 15 mg/kg given intravenously. This dose was found (Guldborg, 1967, p. 108) to produce very little effect on the levels of the acid metabolites of dopamine in the C.S.F. and it was of interest to determine if it had an equally small effect on the levels of the acids and of the amines in the brain tissue. In this series of experiments, the hindbrain was also investigated because chlorpromazine has been postulated to act on the brain stem reticular formation (Bradley and Key, 1959). For technical reasons, the

hindbrain was not included in the series of analyses in the experiments with the lower dose of chlorpromazine.

## METHODS

Dissection and Extraction of Brain Tissues

Male and female beagle dogs of about 10 kg body weight and aged 1 - 2 years, with chronically implanted guide-tubes to allow sampling of lateral ventricular cerebrospinal fluid (C.S.F.) (Guldborg, 1967, p. 37), were used. For the period of the experiment the dogs were kept in a room with a thermostatically controlled environmental temperature of 21°C, the rectal temperature being measured from time to time with a clinical thermometer. The dogs were anaesthetised with intravenous thiopentone sodium B.P. (Pentothal, Abbott Labs.). The plane of anaesthesia, as judged essentially by clinical signs, was kept as uniform as possible from dog to dog. Chlorpromazine (Largactil Inj. B.P., May and Baker) was administered intravenously 5 - 10 min. after induction of anaesthesia, to 4 dogs in a dose of 5 mg/kg and to 4 dogs in a dose of 15 mg/kg, taking 5 min. to inject the requisite amount. Eight dogs, for use as controls, did not receive chlorpromazine. Experiments on control and treated dogs were interspersed to minimise the effect of any technical differences between experiments, on the comparisons of estimates obtained for control and treated animals. The amount of anaesthetic required to maintain anaesthesia in untreated dogs was much more than that required to maintain

TABLE 15

## Delineation of Areas of Dog Brain estimated for Catecholamines and their Metabolites

Dissections based on stereotaxic atlas of Lim, Liu and Moffitt (1960)

Area	Delineation	Mean Weight of Areas dissected from 16 Dogs $\pm$ S.D. (g)	Weight of Area processed (approx.) (g)
Caudate Nucleus	Both nuclei dissected	$1.50 \pm 0.26$	0.3
Globus Pallidus	Section based on coronal section (Lim, Liu and Moffitt, 1960, Fig. 20, R 20) and included globus pallidus and putamen, which were found to lie between cortex and internal capsule, after removal of the thalamus.	group 1: $0.77 \pm 0.29$ group 2: $0.35 \pm 0.12$	0.7 0.4
Hypothalamus	From the optic chiasma anteriorly up to and including the mammillary bodies posteriorly and demarcated superiorly by the thalamus.	$0.76 \pm 0.16$	0.8
Midbrain	That part of the brainstem extending from the hypothalamus up to and excluding the pons.	$2.12 \pm 0.20$	1.0
Cortex	The grey matter of the occipital cortex with as little white matter as possible.	$1.18 \pm 0.23$	1.0
Thalamus	Both thalami dissected	$1.38 \pm 0.28$	1.4
Hindbrain	That part of the brainstem extending from and including the pons anteriorly, to the beginning of the spinal cord.	$4.03 \pm 0.38$ (7 dogs only)	1.0

anaesthesia in the chlorpromazine-treated dogs. A 0.5 ml sample of lateral ventricular C.S.F. was withdrawn just after induction of anaesthesia and before the injection, if any, of chlorpromazine, as described by Guldberg (1967, p. 37). Two further 0.5 ml samples of lateral ventricular C.S.F. were withdrawn at 1 hour and 2 hours respectively, after induction of anaesthesia. The animal was then killed, i.e. after 2 hours anaesthesia, by exsanguination from a femoral vein.

The top of the skull was cut out, using an electric saw, to enable removal of the whole brain, which was then halved sagittally. The following areas, delineated as in Table 15, were dissected and immediately wrapped in aluminium foil and frozen in solid carbon dioxide (p. 94): caudate nucleus, globus pallidus, hypothalamus, midbrain, cortex, thalamus and, in experiments on the effect of 15 mg/kg chlorpromazine, hindbrain. The length of time between killing the animal and freezing these areas varied from 20 - 60 min.. The mean weight and standard deviation of each area are given in Table 15. The weights of the globus pallidus are meaned in two groups, each consisting of 4 untreated dogs and 4 dogs treated with 5 mg/kg or 15 mg/kg chlorpromazine.

This was necessary because there was a significant difference in the weights of this area between the two groups of experiments, the dissections in the two groups having been carried out by different workers. There was no significant between-group difference in the weights of the other areas, suggesting that the delineations of the other brain areas, by the two dissectors were the same.

The whole of each area removed was homogenised in perchloric acid and centrifuged to give a perchloric acid extract, as described on p. 95. If the weight of tissue homogenised was more than 1.4 g, only a portion of this extract, equivalent to about 1g tissue, was processed further, in order to prevent overloading of the chromatogram with tissue extract. Latterly, the weight of caudate nucleus was restricted to about 0.3 g per sample because larger weights contained such large amounts of dopamine that on chromatography a wide acetyl-dopamine band was obtained which obtruded into the neighbouring acetyl-metanephrine and acetyl-methoxydopamine positions.

Two portions were taken of the caudate and midbrain extracts after removal of protein by centrifugation (p. 95) and a mixture of known amounts of the amines and acids added to one portion from each of these extracts, to determine the recoveries of the



amines and acids through the whole procedure. 0.10 ml of a mixture containing 0.5 ug dihydroxyphenylacetic acid and 2 ug homovanillic acid in 0.01N hydrochloric acid was added to portions of the caudate and midbrain extracts. Different amounts of the amines contained in 0.10 ml 0.01N hydrochloric acid were added to these two extracts in order to determine if the recovery of the amines through the procedure was concentration dependent. Thus 0.20 ug noradrenaline, 0.10 ug or 0.20 ug adrenaline and metanephrine, 1.00 ug dopamine and 0.40 ug methoxydopamine were added to the caudate sample and 1.00 ug noradrenaline, 0.40 ug adrenaline and metanephrine and 0.20 ug dopamine and methoxydopamine were added to the midbrain sample. In some experiments, the amines and acids were added to portions of the caudate and midbrain homogenates before centrifugation to determine if the presence of precipitated protein affected the amine and acid recoveries.

#### Extraction of Acid Metabolites into Ethyl Acetate

After the foregoing volume adjustments and preparation of recovery samples, the pH of the extract was adjusted to 4 to precipitate perchlorate as on p. 96 . The extract was then acidified to pH 1 - 2 and shaken with ethyl acetate to remove the acid



metabolites of the amines (p. 97 ). In order to reduce the amount of amine which might be present in the ethyl acetate extract as a result of mechanical carry-over from the aqueous phase, the ethyl acetate fraction was shaken for 5 min. with 2 ml 0.01N hydrochloric acid which had been saturated with sodium chloride. The two phases were separated by centrifugation at about 980 g for 5 min. and the aqueous phase withdrawn. The fact that no adrenaline or noradrenaline was detected in the ethyl acetate fraction after shaking an acid solution of 200 mug amounts of the amines with 1.5 vol. ethyl acetate (p. 121 ), suggested that the amount of amine carried over into ethyl acetate was very small compared with the amount remaining in the aqueous phase. However, even a very small degree of carry-over into ethyl acetate from an extract of caudate nucleus, which contains high concentrations of dopamine (Lavery and Sharman, 1965), could result in sufficient dopamine passing from the ethyl acetate fraction into the 'tris' buffer, to interfere with the estimation of dihydroxyphenylacetic acid. Interference could occur because dopamine and dihydroxyphenylacetic acid form fluorophors with ethylenediamine which have the same wavelength characteristics and the concentration of dopamine in the caudate nucleus is considerably in

excess of dihydroxyphenylacetic acid (Sharman, Poirier, Murphy and Sourkes, 1967). The ethyl acetate extracts obtained from samples of dog brain were therefore washed with acid, as described.

Since the percentage carry-over of adrenaline and noradrenaline had been found to be low (p.121), the acid washes were discarded in the first group of experiments, consisting of 4 control dogs and 4 dogs which had received 5 mg/kg chlorpromazine. After it had been found that a significant amount of noradrenaline was removed into ethyl acetate from acid extracts of hypothalamus (p.198), the acid wash was added to the aqueous amine extract in the second group of experiments of 4 control dogs and 4 dogs treated with 15 mg/kg chlorpromazine. The acid metabolites of the amines were back-extracted from the ethyl acetate fraction into alkaline 'tris' buffer (p.97). Separate portions of this buffer were used for the estimation of homovanillic acid and dihydroxyphenylacetic acid (Guldborg, 1967, p. 39).

#### Estimation of the Amines

The amines were assayed as their acetylated derivatives (p.108-117), with the exception of normetanephrine which could not be included because at the time of these experiments, its method of assay had

failed. Investigations into the reason for this failure are given on p. 132. In order to limit the number of assays and because the method of assay of dopa described on p. 119 was not very sensitive, no attempts were made to estimate dopa in dog brain. A more sensitive method of dopa estimation was subsequently developed (Appendix).

Estimation of Acetyl-methoxydopamine in Eluates  
from Chromatograms of Extracts containing large  
Amounts of Acetyl-dopamine

Certain precautions, unnecessary when eluting amounts of acetyl-dopamine of the order of 0.6 ug such as were obtained on chromatographing extracts equivalent to 1 g rat brain (p. 174), were required for the quantitative elution of the much greater amounts of acetyl-dopamine (about 2 - 3 ug) obtained on chromatographing extracts of 0.3 g caudate nucleus. Investigation of the effect of extracts of brain grey matter on the chromatography of acetyl-dopamine (p. 150) had indicated that, in the presence of such extracts, the band width of 2 - 3 ug acetyl-dopamine on the chromatogram could be expected to be greater than the band width of the visualised marker. Sections of the chromatograms from the dog brain extracts extending from 0.5 cm below the lower limit of the acetyl-metaneprine marker, to the half-way position in the space

between the acetyl-dopamine and acetyl-methoxydopamine visualised markers, were therefore eluted and assayed for acetyl-dopamine. There was usually a 2 cm separation between these two markers (Figure 15, p. 142). However, since with 0.8 - 2 ug amounts of acetyl-dopamine, some was found to run up to 3 cm beyond the farthest limit of the acetyl-dopamine marker (p. 146), a significant amount of acetyl-dopamine could be expected in the acetyl-methoxydopamine eluates from those chromatograms carrying extracts derived from brain areas, such as caudate nucleus and globus pallidus, which contain these high levels of dopamine. The acetyl-methoxydopamine eluates were therefore assayed for acetyl-methoxydopamine and acetyl-dopamine as follows:

A 1.00 ml portion of the eluate was put through the acetyl-methoxydopamine assay technique (p. 110), which assays both acetyl-methoxydopamine and acetyl-dopamine (p. 129). Standard amounts of acetyl-dopamine (100 - 400 mug) were processed through the acetyl-methoxydopamine assay in order to obtain a measure, which varied slightly from run to run (p. 130), of the relative fluorescence intensities of these two substances. A second eluate portion, 0.80 ml, was assayed for acetyl-dopamine alone (p. ), by the method given on p. 108. With this information it

was possible to calculate the contribution made by acetyl-dopamine to the fluorescence reading obtained by the acetyl-methoxydopamine assay, and hence, to calculate the fluorescence due to acetyl-methoxydopamine.

Investigations into the Transfer of Acetylated Amines  
from round-bottomed Flasks to Paper Chromatograms

Incomplete transfer of amines, from the round-bottomed flask in which the dichloromethane was evaporated, to the paper chromatogram (p. 99) was anticipated as a possible source of amine loss. This possibility was examined as follows. After application of the flask contents to the chromatogram, 3 ml water were added to the flask and shaken vigorously to effect solution of any acetylated amines adhering to the flask wall. A 2.0 ml portion was treated with ethylenediamine/ammonium chloride as on p. 108 and the relative fluorescence read at the wavelengths of maximal fluorescence of acetyl-noradrenaline and acetyl-dopamine (430/500 mμ and 430/520 mμ, respectively). A fluorescence with the wavelength characteristics of acetyl-noradrenaline, if the sample was derived from an area containing predominantly noradrenaline and with the wavelength characteristics of acetyl-dopamine, if the sample was derived from an area containing predominantly dopamine and equivalent to

about 15% of the amount of amine recovered from the chromatogram, was obtained from round-bottomed flasks used in three preliminary experiments, in which the wash-down technique with dichloromethane (p. 99 ) was omitted and the methanol was introduced into the flask without being allowed to flow down the flask wall.

Following these results, wash-down of the flask wall with dichloromethane (p. 99 ) and solution in methanol, of the residue obtained by evaporation of the dichloromethane extract, using the precautions given on p. 99 , were incorporated into the routine experimental procedure. The efficiency of these techniques in facilitating transfer of the amines to the paper chromatogram was checked by testing the round-bottomed flasks used in four experiments, selected at random, for the presence of the amines (p. 194 ). The fluorescence readings obtained from flasks which had contained extracts of globus pallidus, midbrain, hypothalamus, thalamus and hindbrain were found to be the same as the corresponding readings from flasks which had contained extracts of cortex and about 1.5 times the fluorescence readings of the reagent blanks. It can be concluded that no amines were present in these flasks and that transfer of the amines in extracts of globus pallidus, midbrain,



hypothalamus, thalamus and hindbrain, to the paper chromatogram, was quantitative. A small but significant amount of fluorescence, showing maximal intensity at wavelengths characteristic of the acetyl-dopamine fluorophor and equivalent to 80 - 160 mug acetyl-dopamine or  $4\% \pm 4\%$  (S.D.) of the acetyl-dopamine recovered from the chromatogram, was found in flasks which had contained extracts of caudate nucleus or extracts of caudate nucleus to which dopamine had been added (p.189). Since this was a low and reasonably constant percentage loss, it was ignored when calculating the acetyl-dopamine concentration of tissue samples.

Detection of partially Acetylated Amines  
at the Origins of Chromatograms of Extracts of Dog  
Brain

In order to check for deposition at the origin of a chromatogram, of any partially acetylated catecholamines (p.153) a band extending from 1 cm behind, to 2 cm in front of, the line of application, was cut from each chromatogram, eluted in 3 ml water and a 2.0 ml portion treated with ethylenediamine/ammonium chloride as on p.168.

The fluorescence produced from the origin eluates from chromatograms of extracts of cortex,



thalamus, hindbrain, midbrain and hypothalamus did not differ significantly from that produced from the paper blank, implying that the acetylation and chromatography procedures had not resulted in the formation of detectable amounts of partially acetylated catecholamines in these extracts.

A fluorescence showing maximal intensity at the wavelengths characteristic of maximal fluorescence of acetyl-dopamine (i.e. 430/520 mμ) and equivalent to  $2\% \pm 2\%$  (S.D.) of the acetyl-dopamine recovered from the acetyl-dopamine position, was produced from the origin eluates of chromatograms of 36 extracts containing large amounts (i.e. more than 750 mμg) of acetyl-dopamine. These extracts comprised extracts of caudate nucleus, caudate nucleus to which 1 μg dopamine had been added (p.189) and globus pallidus. The most probable reason for the presence of this fairly uniform percentage of acetyl-dopamine at the origin would appear to be that the acetylation procedure gave rise to a small, but constant proportion of incompletely acetylated dopamine, probably N-acetyl-dopamine (p.164). This incompletely acetylated dopamine, since it accounted for only  $2\% \pm 2\%$  (S.D.) of the total acetyl-dopamine, was ignored when calculating the acetyl-dopamine content of the tissue extracts. Lavery and Sharman (1965) found

0.5% of the total acetyl-dopamine in an extract could be recovered from the acetyl-noradrenaline position. Since our chromatograms were developed for a longer time than those of Lavery and Sharman, we obtained complete separation of this incompletely acetylated dopamine from acetyl-noradrenaline, on the few occasions, always when gross de-acetylation was known to have occurred (p.153 ), on which it was detected on the chromatogram.

Presence of Acetyl-dopamine and Acetyl-noradrenaline,  
in the Acid Washes of Ethyl Acetate Extracts of  
Perchloric Acid Extracts of Caudate Nucleus and  
Hypothalamus respectively

The estimates for noradrenaline in hypothalamus and midbrain obtained in the first group of control dogs were, like those obtained for noradrenaline in rat brain using the same technique, about half the corresponding estimates quoted in the literature (Table 16 , p.201 , Table 22 , p.228). Estimates for noradrenaline in rat brain agreeing with those reported in the literature were obtained in experiments in which the acid extract of brain was not extracted with ethyl acetate (p.84 ). These findings suggested that, in the presence of an extract of brain tissue, noradrenaline may be removed from an acid,salt-saturated

solution by ethyl acetate.

The ethyl acetate fraction was shaken for 4 min. with 2 ml 0.01N hydrochloric acid saturated with sodium chloride and after centrifugation to separate the layers, the aqueous phase was removed by pipette and treated with ethylenediamine/ ammonium chloride as on p. 108.

A material fluorescing maximally at 430/520 mμ for the caudate nucleus acid wash and at 430/500 mμ for the hypothalamus and cortex acid washes was present in these solutions. Since the acid and alcohol metabolic derivatives of the catechol- and methoxy-amines are unlikely to be back-extracted from ethyl acetate into 0.1 vol. acid, dopamine and noradrenaline are the only catechols which might be present in the acid wash and which, on coupling with ethylenediamine, would give rise to fluorophors showing maximal fluorescence at 430/520 mμ and 430/500 mμ, respectively. Using the cortex acid wash as the blank, the acid washes of the ethyl acetate extracts from three samples of caudate nucleus were found to contain dopamine equivalent to 3%, 3% and 1% of the dopamine present in these tissues. The acid washes from three samples of hypothalamus were found to contain noradrenaline equivalent to 32%, 30% and 25% of the noradrenaline present in the respective

hypothalami. The cortex acid wash was used as the blank since in the cortical tissue extract the amount of noradrenaline was found to be about  $\frac{1}{2}$  of that in the hypothalamus tissue extract and the amount of dopamine about  $\frac{1}{20}$  of that in the caudate nucleus tissue extract.

These results indicated that a significant proportion of the tissue noradrenaline, but not dopamine, was removed into ethyl acetate and could be recovered from this ethyl acetate, by back-extraction into acid solution. In the second group of dog experiments, the acid wash from the ethyl acetate fraction was therefore added to the aqueous amine extracts (p. 191) before the acetylation stage of the procedure.

The concentrations of catecholamines and their metabolites in discrete areas of the brains of control dogs and of dogs treated with chlorpromazine (CPZ) 5 mg/kg intravenously 2 hr. before killing.

Concentrations<sup>\*</sup> given in  $\mu\text{g/g}$  tissue (mean  $\pm$  standard deviation for estimates from four experiments unless otherwise indicated by number in parenthesis. Uncorrected for recoveries.)

	Noradrenaline	Adrenaline	Metanephrine	Dopamine	Methoxy-dopamine	Homovanillic acid	Dihydroxy-phenylacetic acid
	Control CPZ	Control CPZ	Control CPZ	Control CPZ	Control CPZ	Control CPZ	Control CPZ
Caudate nucleus	<0.20 (3)  0.13 (3) $\pm 0.02$	<0.03  <0.04	<0.05 (3)  <0.04	5.5 $\pm 1.4$  5.6 $\pm 1.1$	0.38 (3) $\pm 0.24$  0.07 (3) $\pm 0.16$	13.4 $\pm 1.14$  17.0 $\pm 1.08$	1.3 $\pm 0.27$  1.9 $\pm 0.4$
Globus Pallidus	0.11 $\pm 0.08$  0.08 (3) $\pm 0.05$	<0.05  <0.04	<0.04 (3)  <0.03	0.54 $\pm 0.3$  0.46 $\pm 0.19$	0.10 $\pm 0.04$  0.07 (3) $\pm 0.05$	3.9 $\pm 0.8$  2.85 $\pm 2.05$	0.17 (3) $\pm 0.03$  0.29 (2) $\pm 0.3$
Hypo-thalamus	0.40 $\pm 0.12$  0.60 $\pm 0.19$	0.08 $\pm 0.03$  0.06 $\pm 0.02$	<0.05  0.03 $\pm 0.01$	0.15 $\pm 0.05$  0.22 $\pm 0.04$	<0.06  0.02 (2) $\pm 0.03$	2.5 (3) $\pm 0.1$  2.35 $\pm 0.16$	0.09 (3) $\pm 0.03$  0.33 (3) $\pm 0.25$
Midbrain	0.22 $\pm 0.06$  0.31 $\pm 0.12$	<0.04  <0.03	<0.03 (3)  <0.03	0.12 $\pm 0.05$  0.12 $\pm 0.04$	0.02 (3) $\pm 0.02$  0.02 (2) $\pm 0.01$	1.8 $\pm 0.3$  1.45 $\pm 0.17$	0.05 (3) $\pm 0.03$  0.12 (2) $\pm 0.06$
Cortex	0.04 $\pm 0.03$  0.06 $\pm 0.05$	<0.02  <0.03	<0.03 (3)  <0.02	0.08 $\pm 0.03$  0.03 (2) $\pm 0$	<0.04  0.02 (2) $\pm 0$	Not done	Not done
Thalamus	0.13 $\pm 0.02$  0.24 $\pm 0.06$	<0.03  <0.03	<0.03 (3)  <0.02 (3)	0.03 $\pm 0.01$  0.13 $\pm 0.06$	<0.03  0.02 (3) $\pm 0.01$	1.65 $\pm 0.4$  1.98 $\pm 0.26$	0.06 (2) $\pm 0.03$  0.06 (2) $\pm 0.05$

\* Acid back washes from ethyl acetate extracts were not added to the amine containing fraction in this series of experiments (p. 191).

The concentrations of homovanillic acid and dihydroxyphenylacetic acid are quoted from the results obtained by Dr H.C. Guldberg.

! < ' estimates obtained from four experiments unless otherwise indicated by number in parenthesis.



The concentrations of catecholamines and their metabolites in discrete areas of the brains of control dogs and of dogs treated with chlorpromazine (CPZ) 15 mg/kg intravenously 2 hr. before killing.

Concentrations<sup>2</sup> given in µg/g tissue (mean ± standard deviation for estimates from four experiments unless otherwise indicated by number in parenthesis. Uncorrected for recoveries.)

	Noradrenaline		Adrenaline		Metanephrine		Dopamine		Methoxy-dopamine		Homovanillic acid		Dihydroxy-phenylacetic acid	
	Control	CPZ	Control	CPZ	Control	CPZ	Control	CPZ	Control	CPZ	Control	CPZ	Control	CPZ
Caudate nucleus	<0.18 (3)	0.29 (3) ±0.18	<0.04	<0.06	<0.05 (2)	<0.03 (2)	5.5 ±1.6	3.8 ±0.4	0.61 ±0.30	0.17 (3) ±0.05	12.5 ±1.07	12.48 ±0.9	1.03 (3) ±0.12	0.94 ±0.3
Globus Pallidus	<0.19 (3)	0.18 (3) ±0.06	<0.04	<0.06	<0.07	<0.03 (2)	0.81 ±0.42	0.27 (3) ±0.21	0.09 ±0.04	<0.08 (3)	2.2 ±1.2	2.24 (3) ±0.7	Not detected	
Hypo-thalamus	0.59 ±0.22	0.86 ±0.34	0.05 ±0.02	0.05 (3) ±0.05	<0.02 (3)	<0.01 (2)	0.21 ±0.03	0.16 ±0.05	<0.04	<0.04 (3)	1.1 ±0.4	1.14 ±0.22	0.18 (1)	0.21 (2) ±0
Midbrain	0.44 ±0.21	0.44 ±0.15	<0.02	<0.02	<0.02	<0.01 (3)	0.14 ±0.04	0.12 ±0.07	0.02 (2) ±0.02	<0.03 (3)	1.04 ±0.4	1.17 ±0.5	0.16 (1)	0.17 (2) ±0
Cortex	0.08 (3) ±0.01	<0.12	<0.02	<0.02	<0.02	<0.01 (3)	<0.04	<0.04	<0.02	<0.03 (3)	Not done	Not done	Not done	Not done
Thalamus	0.20 ±0.07	0.41 (3) ±0.06	<0.02	<0.02 (2)	<0.02	<0.01 (3)	0.04 ±0.01	0.04 ±0.02	<0.02	<0.02 (3)	1.06 ±0.3	1.28 ±0.4	0.18 (1)	0.10 (1)
Hind-brain	0.28 (3) ±0.07	0.43 (3) ±0.11	<0.02 (3)	<0.02 (3)	<0.02 (3)	<0.01 (3)	0.02 (3) ±0.01	0.02 (3) ±0.01	<0.02 (3)	<0.02 (3)	0.17 (3) ±0.12	0.24 ±0.05	Not detected	

<sup>2</sup>Acid back washes from ethyl acetate were added to the amine containing fraction in this series of experiments (p. 191 ).  
The concentrations of homovanillic acid and dihydroxyphenylacetic acid are quoted from the results obtained by Dr H.C. Guldberg.  
'<' estimates obtained from four experiments unless otherwise indicated by number in parenthesis.

## RESULTS

Effect of Chlorpromazine on the Levels of the  
Catecholamines and Their Metabolites in discrete  
Areas of Dog Brain

Estimates of noradrenaline, adrenaline, metanephrine, dopamine, methoxydopamine, homovanillic acid and dihydroxyphenylacetic acid in the caudate nucleus, globus pallidus, hypothalamus, midbrain, cortex and thalamus from 4 control dogs and from 4 dogs treated with intravenous chlorpromazine (5 mg/kg) 2 hr. before sacrifice are given in Table 16 .

Estimates of the same amines and acids in these six areas of brain and also in the hindbrain, from 4 control dogs and 4 dogs treated with intravenous chlorpromazine (15 mg/kg) 2 hr. before sacrifice are given in Table 17 . The two groups of controls were necessary because after the first 4 control dogs and 4 dogs treated with 5 mg/kg chlorpromazine, the technique was slightly modified and the dissections performed by a different person (p.188). The mean procedure recovery of homovanillic acid and of dihydroxyphenylacetic acid from twelve experiments was 99% and 107%, respectively. No correction for recovery of the homovanillic acid and dihydroxyphenylacetic acid estimates was therefore necessary (Guldborg, personal communication). The procedure recoveries of the amines, however, varied widely, and did not appear



TABLE 18

Effect of Chlorpromazine (5 mg/kg and 15 mg/kg) 1/V 2 hr. before sacrifice) on the Levels of the Catecholamines and their Metabolites in discrete Areas of Dog Brain.

	Noradrenaline 15 mg/kg 5 mg/kg	Dopamine 15 mg/kg 5 mg/kg	Methoxy- dopamine 15 mg/kg 5 mg/kg	Homovanillic acid 15 mg/kg 5 mg/kg	Dihydroxyphenyl- acetic acid 15 mg/kg 5 mg/kg
Caudate nucleus	0 0	↓(<0.10) 0	↓(<0.05) ↓(<0.01)	0 ↑(<0.01)	0 ↑(<0.05)
Globus Pallidus	0 ↓ns	↓(<0.10) 0	↓ns ↓ns	0 ↓ns	ND ↑ns
Hypo- thalamus	↑ns ↑ns	↓ns ↑ns	0 0	0 0	0 ↑ns
Midbrain	0 ↑ns	0 0	0 0	0 ↓ns	0 ↑ns
Cortex	0 ↑ns	0 ↓ns	0 0	ND ND	ND ND
Thalamus	↑(<0.01) ↑(<0.01)	0 ↑(<0.02)	0 0	↑ns ↑ns	↓ns 0
Hindbrain	↑ns ND	0 ND	0 ND	↑ns ND	ND ND

0 = no alteration in mean value from control

↑ = mean value greater than that of control

↓ = mean value less than that of control

ns = difference between mean values of 'drug' and control group not significant ( $p > 0.1$ )

( ) = probability level of difference between 'drug' and control group being due to chance

ND = not done

to reflect the recoveries of the corresponding endogenous amines (p. 218). The amine estimates were therefore not corrected for procedure recoveries.

The estimates for noradrenaline, dopamine, methoxydopamine, homovanillic acid and dihydroxyphenylacetic acid in each area from the brains of dogs which had been treated with chlorpromazine were compared with the corresponding estimates from the brains of control dogs in the same group of experiments, using the Student's 't-test' (Table 18). The concentrations of methoxydopamine in areas other than the caudate nucleus and the globus pallidus were at or below the limit of detection of the method. The estimate of 0.38  $\mu\text{g/g}$  for methoxydopamine in the caudate nucleus in the first control group of experiments must be regarded with suspicion because at the time of these experiments, it was not realised that the ratio of the fluorescence intensities derived from equimolar amounts of acetyl-methoxydopamine and acetyldopamine using the acetyl-methoxydopamine method of assay, could vary from run to run (p. 130). Comparisons were therefore made using the estimate of 0.61  $\mu\text{g/g}$ , obtained in the second group of control experiments. The hypothalamus was the only area in which adrenaline was found. Neither dose of chlorpromazine affected the levels of hypothalamic adrenaline. Metanephrine reached just detectable levels

Figure 19

Fluorescence Spectra from the 'Acetyl-dopamine' Eluate from Chromatograms of Extracts of Dog Globus Pallidus and Cortex and their corresponding Paper Blank, as compared with the Fluorescence Spectra from authentic Acetyl-dopamine and the Reagent Blank.

Excitation wavelength - 430 m $\mu$ .

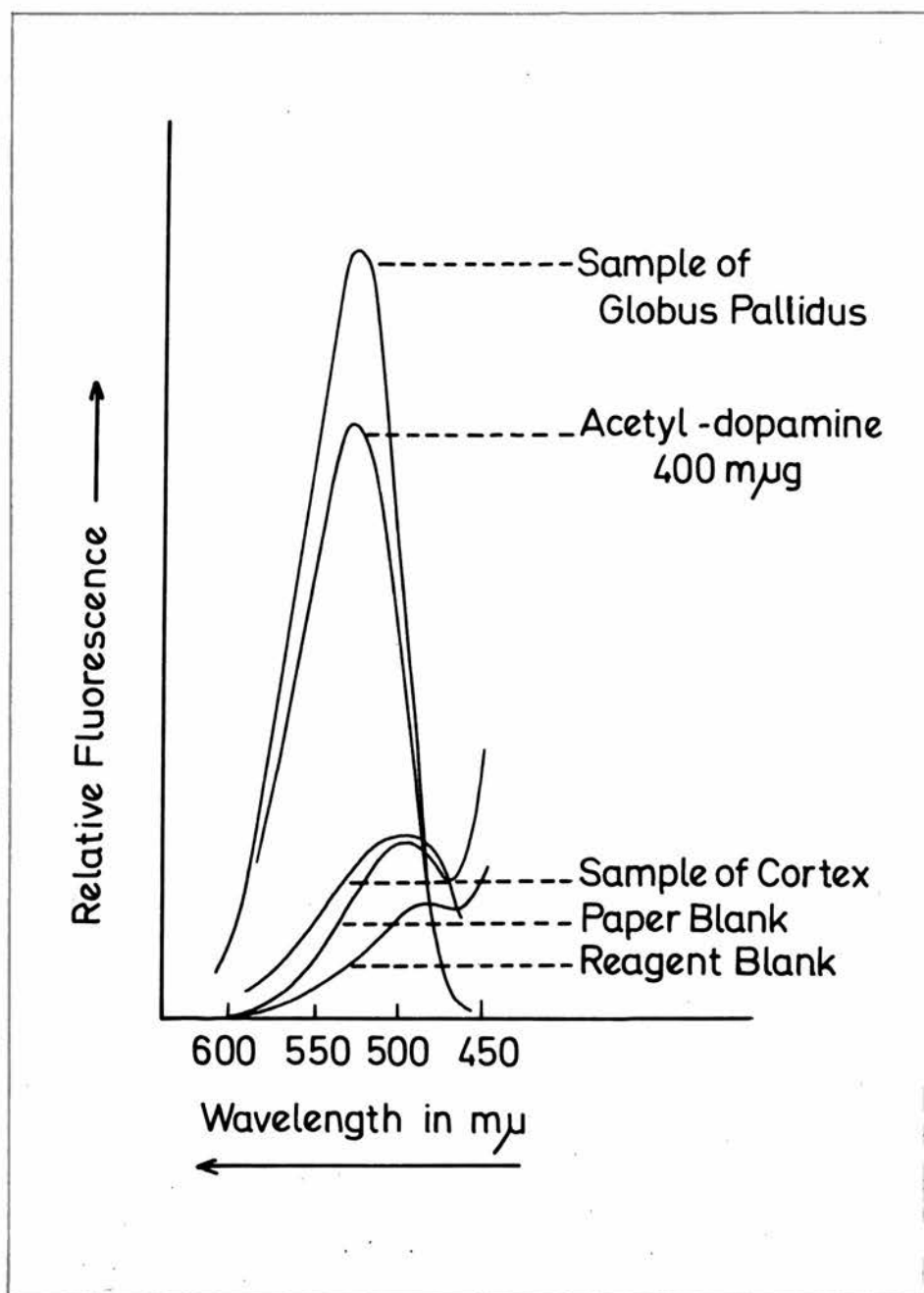
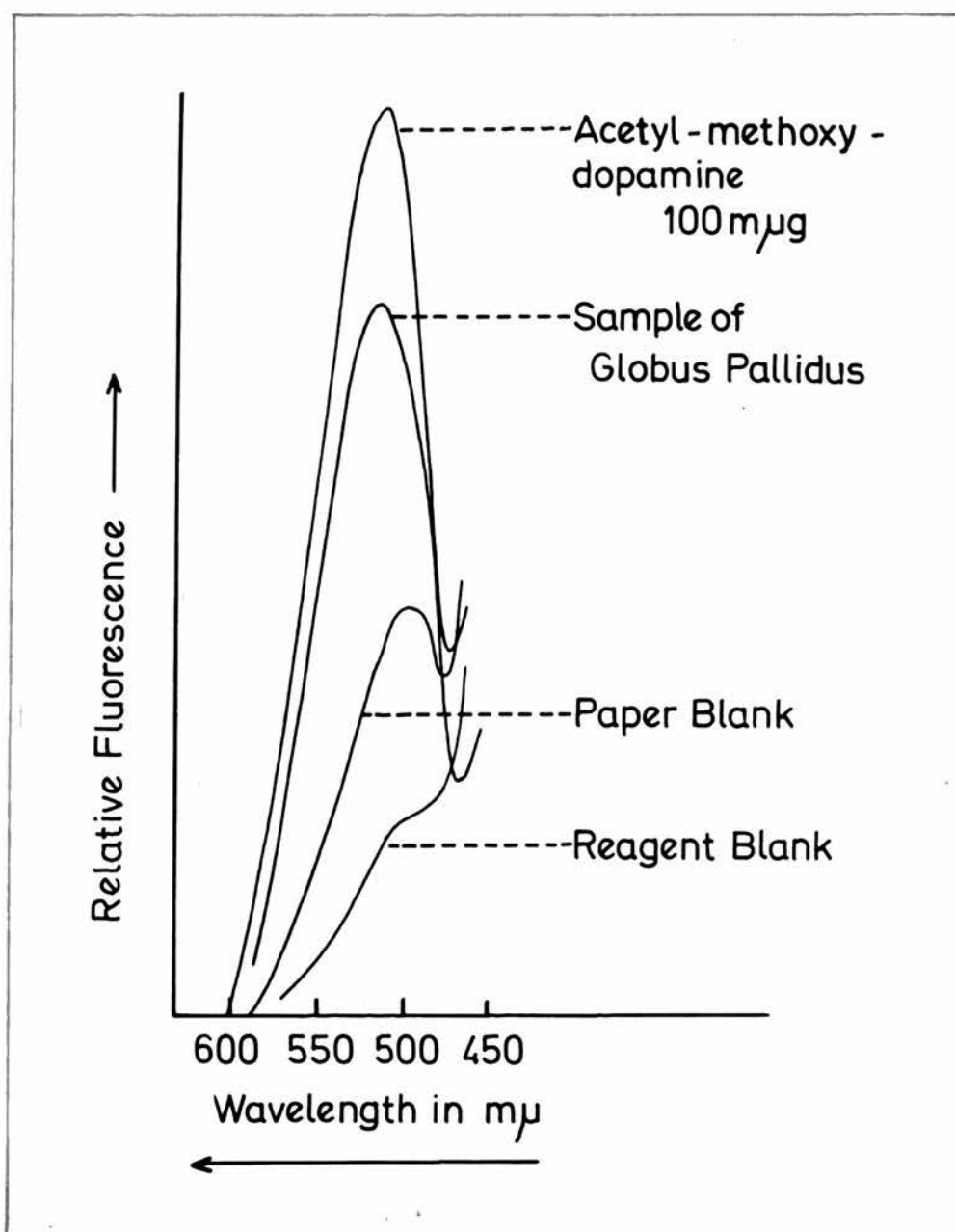


Figure 20

Fluorescence Spectra from the 'Acetyl-methoxydopamine' Eluates from Chromatograms of an Extract of Dog Globus Pallidus and its corresponding Paper Blank, as compared with the Fluorescence Spectra from authentic Acetyl-methoxydopamine and the Reagent Blank.

Excitation wavelength - 430 m $\mu$ .



in the extracts of hypothalamus from 4 dogs which had received 5 mg/kg chlorpromazine but the concentrations were not significantly different from the controls. No metanephrine was found in any other area. No dihydroxyphenylacetic acid was detected in extracts of globus pallidus and hindbrain from dogs treated with 15 mg/kg chlorpromazine and from control dogs in the same group of experiments (Table 17 ).

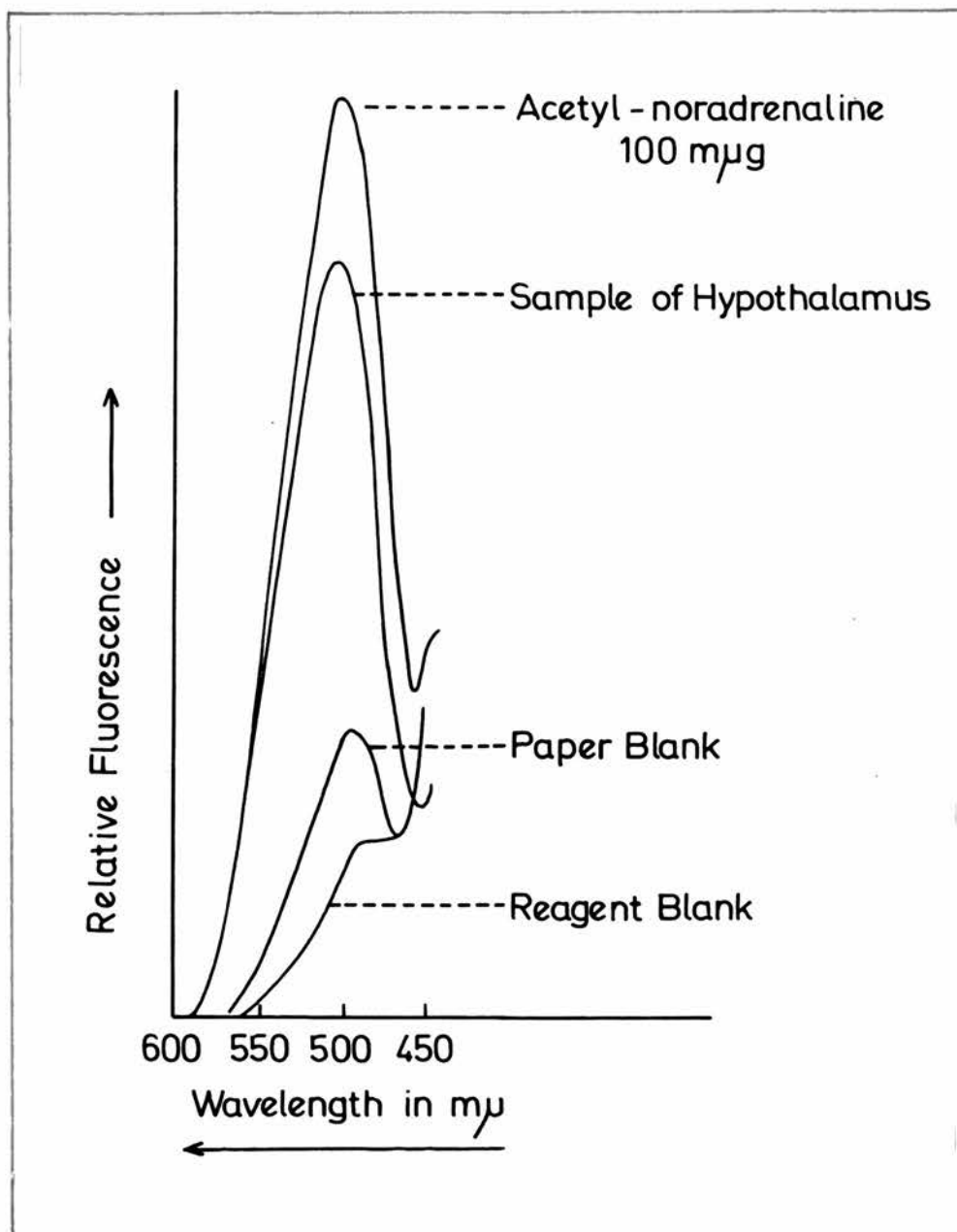
Fluorescence Characteristics of Eluates from Chromatograms of Dog Brain Extracts

The 'acetyl-dopamine' and 'acetyl-methoxydopamine' eluates from chromatograms of tissue extracts containing measurable concentrations of dopamine and methoxydopamine produced fluorophors which had the same wavelength characteristics as the fluorophors from authentic dopamine and methoxydopamine (Figures 19 and 20 ). As little as 20  $\mu$ g acetyl-dopamine and 25  $\mu$ g acetyl-methoxydopamine produced a fluorescence which was maximal at 430/520  $m\mu$  and was distinguishable from the fluorescence from the paper blank, which was maximal at 430 - 440/485 - 495  $m\mu$ . The fact that the fluorescence spectrum from the 'acetyl-dopamine' eluate from a chromatogram of an extract of cortex was the same as the spectrum from the corresponding paper blank (Figure 19 ) demonstrates that the extract

Figure 21

Fluorescence Spectra from the 'Acetyl-noradrenaline' Eluates from Chromatograms of an Extract of Dog Hypothalamus and its corresponding Paper Blank, as compared with the Fluorescence Spectra from authentic Acetyl-noradrenaline and the Reagent Blank.

Excitation wavelength - 430 m $\mu$ .



of cortex contained no detectable acetyl-dopamine. The 'acetyl-noradrenaline' and 'acetyl-adrenaline' eluates from chromatograms of extracts containing measurable amounts of noradrenaline and adrenaline gave rise to fluorophors which showed maximal fluorescence at wavelengths characteristic of the fluorophors from authentic acetyl-noradrenaline (Figure 2/ ) and acetyl-adrenaline. Unfortunately, since the fluorophors from acetyl-noradrenaline and acetyl-adrenaline fluoresced maximally at wavelengths (430/500 mμ) close to those of maximal fluorescence of the paper blank (430 - 440/485 - 495 mμ), it was not possible to differentiate the fluorescence due to the blank from that due to the acetylated amine. Extracts of hypothalamus from 4 dogs treated with 5 mg/kg chlorpromazine were the only extracts in which metanephrine was detected. Since there was only about 10 - 20 mμg acetyl-metanephrine in the portion of the 'acetyl-metanephrine' eluate taken for assay, no fluorescence peak was obtained at the wavelengths of maximal fluorescence of acetyl-metanephrine except in one sample in which there was a slight fluorescence peak at 520 mμ although there was no indication of an excitation peak at 420 mμ. Eluates from a chromatogram of an extract of caudate nucleus to which known amounts of the amines had been added (p. 188) produced



fluorophors which showed maximal intensity at wavelengths characteristic of the fluorophor of the acetylated amine they were expected to contain.

Effect of Eluates from Chromatograms of Brain Extracts from Dogs which had received Chlorpromazine, on the Sensitivities of Estimation of the Acetylated Amines

Although quenching of the fluorescence of added acetylated amines by eluates from chromatograms of extracts of rat brain was negligible (p. 109, III, 117), it is possible that extracts of brains from dogs pretreated with chlorpromazine might contain chlorpromazine or a metabolite of chlorpromazine which might affect the fluorescence from the acetylated amines. The fluorescence intensities from 1  $\mu$ g and 100  $\mu$ g acetyl-dopamine added to 1 ml portions of the 'acetyl-dopamine' eluates from chromatograms of extracts of a caudate nucleus and a midbrain, respectively, which had originated from a dog treated with 15 mg/kg chlorpromazine, were the same as those derived from aqueous standards of acetyl-dopamine. The fluorescence from 100  $\mu$ g acetyl-noradrenaline was similarly unaffected by the presence of a 1 ml portion of the 'acetyl-noradrenaline' eluates from chromatograms of two extracts of midbrain from 2 dogs treated with 15 mg/kg chlorpromazine. The fluorescence from 100  $\mu$ g acetyl-methoxydopamine was unaltered by the presence of a 1 ml

portion of the 'acetyl-noradrenaline' eluates from chromatograms of two extracts of midbrain from 2 dogs treated with 15 mg/kg chlorpromazine. The fluorescence from 100 mug acetyl-methoxydopamine was unaltered by the presence of a 1 ml portion of the 'acetyl-methoxydopamine' eluates from chromatograms of extracts of caudate nucleus and globus pallidus from a dog treated with 15 mg/kg chlorpromazine. Thus, if chlorpromazine or any of its metabolites were present in the extract applied to the chromatogram, they did not interfere with the fluorophor production or measurement of the fluorescence from, acetyl-noradrenaline, acetyl-dopamine or acetyl-methoxydopamine.

The following observations indicated that neither chlorpromazine nor its metabolites contributed to the 'blank' fluorescence in assays of eluates from chromatograms of extracts from chlorpromazine treated dogs:

(i) The relative fluorescence from 'acetyl-noradrenaline' eluates from chromatograms of extracts of globus pallidus was the same as that from the corresponding paper blank irrespective of whether the extracts originated from control or from chlorpromazine (15 mg/kg) treated dogs. Chlorpromazine or its metabolites were therefore unlikely to contribute to the fluorescence from 'acetyl-noradrenaline' eluates from

TABLE 19

Lowest detectable Concentrations of Catecholamines and Methoxyamines in different Areas of Dog Brain ( $\mu\text{g/g}$ ).

Calculated assuming the limit of detection equalled half the sensitivity.

	Noradrenaline	Adrenaline	Metanephrine	Dopamine	Methoxy-dopamine
Caudate Nucleus	0.22	0.07	0.04	0.10	0.07
Globus Pallidus	group 1	0.09	0.02	0.04	0.03
	group 2	0.19	0.03	0.09	0.06
Hypothalamus	0.09	0.03	0.02	0.04	0.03
Midbrain	0.06	0.02	0.01	0.03	0.02
Cortex	0.06	0.02	0.01	0.03	0.02
Thalamus	0.05	0.02	0.01	0.03	0.02
Hindbrain	0.05	0.02	0.01	0.03	0.02

Lowest detectable concentrations in the caudate nucleus are about 3 times greater than in any other area because the weight of caudate nucleus processed was only about  $\frac{1}{3}$  the weight of the other areas processed.

chromatograms of extracts of other brain areas.

(ii) The relative fluorescence from 'acetyl-dopamine' eluates from extracts of cortex was the same as the relative fluorescence from the paper blank, regardless of whether or not the animals from which the extracts originated had been treated with chlorpromazine.

Since there was no evidence that chlorpromazine treatment of the dog resulted in the production of chromatogram eluates which had 'blank' fluorescences different from controls or which produced quenching of the fluorescence from the acetylated amines, the assay sensitivities of the acetylated amines in the presence of eluates from chromatograms of extracts from chlorpromazine treated dogs can be assumed to be the same as those from control animals (Table 19 ).

Using the values for the sensitivity of assay of each acetylated amine in the presence of a chromatogram eluate (Table 13, p. <sup>157</sup>~~213~~) and the weights of each area processed (Table 15, p. 186), the lowest detectable concentration of each amine in each area was calculated (Table 19 ). The limit of detection was taken as equal to half the sensitivity (p. 31 ). Variations in the lowest detectable concentrations of one amine in different areas were due to differences in the amounts of each area processed (Table 15, p. 186 ). Two estimates are given for the globus pallidus, since

TABLE 20

Effect on the levels of noradrenaline in different areas, of combining the acid wash of the ethyl acetate fraction with the amine fraction.

Experiments in group 1 did not have acid wash added to the amine fraction.

Experiments in group 2 had the acid wash added to the amine fraction.

	Noradrenaline in expts. in Group 1	Noradrenaline in expts. in Group 2	p calculated by 't-test'
Caudate Nucleus	< 0.20	< 0.18	
Globus Pallidus	0.11 ±0.08	< 0.19	
Hypothalamus	0.40 ±0.12	0.59 ±0.22	< 0.5
Midbrain	0.22 ±0.06	0.44 ±0.21	< 0.1
Cortex	0.04 ±0.03	0.08 ±0.01	< 0.05
Thalamus	0.13 ±0.02	0.20 ±0.07	≈ 0.1

\* ~~only one estimate~~

the weights of this tissue in experimental groups (1) and (2) were significantly different.

Effect on the Estimates of Noradrenaline in different Areas, of combining the Acid Wash of the Ethyl Acetate Fraction with the Amine Fraction

The means of estimates for noradrenaline in the hypothalamus, midbrain, cortex and thalamus were 32 - 50% greater in 4 control dogs (experiments in group 2) in which the acid wash from the ethyl acetate fraction was combined with the aqueous amine fraction (p. 191), than in 4 control dog experiments (in group 1) in which this acid wash was discarded (p. 191). The estimates for noradrenaline in the hypothalamus and thalamus obtained from the control experiments in group 2 were not, however, significantly different, statistically, from the corresponding estimates obtained from the control experiments in group 1, because of the wide scatter of the individual estimates for each area in both groups of experiments (Table 19). The estimates for noradrenaline in the midbrain and cortex were significantly greater in the control experiments in group 2 than in group 1 ( $p < 0.1$  and  $p < 0.05$ , respectively). The mean of the estimates for dopamine in the caudate nucleus, which contained more dopamine than any other area, were the same



Percent Recoveries of Catechol- and Methoxy-amines added to Samples of Caudate Nucleus and Midbrain at different stages in the Procedure.

All additions made at room temp., unless otherwise indicated.

Stage of addition of amines to sample of caudate nucleus:	Amount of amine added:				
	Noreadrenaline	Adrenaline	Metanephrine	Dopamine	Methoxy-dopamine
(1) just before acetylation	200 mg	100-200 mg	100-200 mg	1 µg	200-400 mg
(2) acid extract just before extraction with ethyl acetate	128	110	97	53	68
(3) extract at pH 4 before KClO <sub>4</sub> removed	120	75	92	98	69
(4) extract before pH to 4, at 0°C (2 experiments)	68	108	59	87	70
(5) extract before pH to 4. (n = 4)	100, 128	88, 62	140, 94	100, 112	136, 44
(6) tissue homogenate (n = 4)	52 ± 17	46 ± 11	37 ± 13	81 ± 26	15 ± 10
	40 ± 54	34 ± 31	71 ± 35	21 ± 23	not done
Amount of amine added:	1 µg	400 mg	400 mg	200 mg	200 mg
Stage of addition of amines to sample of midbrain:					
(5) extract before pH to 4. (n = 4)	44 ± 12	45 ± 5	56 ± 15	53 ± 11	44 ± 8
(6) tissue homogenate (n = 4)	62 ± 48	42 ± 22	58 ± 35	61 ± 41	19 ± 16



whether or not the acid wash was included, suggesting that no significant amount of dopamine was lost into the ethyl acetate. Further evidence of this was provided by the fact that there was no significant difference in the dopamine values for each of the other areas of the brain in the one control group, as compared with the other control group, of experiments, with the possible exception of dopamine in the hypothalamus, which at a 10% level of significance was greater in those samples in which the acid wash was included. Thus it would appear that noradrenaline, but not dopamine, was extracted from an acid extract of brain tissue into ethyl acetate and then back-extracted from the ethyl acetate into 0.01N hydrochloric acid saturated with sodium chloride (p.198).

Recoveries of the Catechol- and Methoxy-amines  
through the Procedure

As was found with rat brain homogenates (p.81 ), recoveries of the amines added to perchloric acid homogenates of caudate nucleus and midbrain in 4 experiments (p.188) were low and very variable (Table 21 ). In contrast, the estimates for the endogenous brain amines were much more consistent. The protein precipitate must have been responsible for some of this variability, since the recoveries

of amines added to the supernatant, obtained by removing the protein precipitate from the homogenate (p.188), although still low, had smaller scatters than the recoveries of amines added to the homogenate (Table 21 ). No obvious difference was obtained between the recoveries of amines added to homogenates and supernatants from the caudate nucleus and the recoveries added to homogenates and supernatants from the mid-brain (Table 21 ). These results were obtained from the first group of dog experiments, in which the acid wash from the ethyl acetate was not included in the amine extract (p.191 ).

The following experiments were designed to determine the exact point of loss of the exogenous amines. Known amounts of the amines (100 mpg - 1 µg) in 0.01N hydrochloric acid were added, at different stages in the procedure, to the portion of the perchloric acid extract of caudate nucleus taken for estimation of the recoveries (p.188 ). In these experiments, the perchloric acid extracts were extracted with ethyl acetate and the acid washes from the organic phases were added to the amine-containing aqueous phases. The stages at which the exogenous amines were added were as follows:

(1) to the extract immediately before the acetylation procedure (p.98 );

- (2) to the acid extract immediately before extraction with ethyl acetate (p. 97 );
- (3) to the extract at pH 4 immediately before storage in the deep-freeze to aid precipitation of potassium perchlorate (p. 96 );
- (4) to the frozen supernatant of the centrifuged perchloric acid homogenate contained in a 15 ml glass stoppered test-tube. The extract was allowed to thaw, almost but not completely, with occasional inversion of the tube to mix in the added amines. The pH was adjusted to 4, as described on p. 96 , using potassium hydroxide solutions at 4°C and intermittently returning the test-tube to the solid carbon dioxide to ensure its contents were maintained at about 0°C, as gauged by the continuous presence of a little ice in the test-tube. The test-tube was then immediately placed in the deep-freeze and processed as on p. 96 .

The results of these experiments prove that variable loss of added amines occurred either on their addition to the perchloric acid extract or during adjustment of the pH of this extract from 0.8 to 4, if these steps were performed at room temperature. Amine loss could be prevented by adding the amines to the frozen extract and carrying out the subsequent pH adjustment at about 0°C.

Homovanillic acid and dihydroxyphenylacetic acid added to 0.4N perchloric acid extracts of caudate nucleus and midbrain (p. /88) at room temperature, were quantitatively recovered without chilling the extract during the pH adjustment (Guldberg, personal communication).

## DISCUSSION

Extraction of Noradrenaline into Ethyl Acetate  
from Acid Extracts of Brain Tissue

Addition of the acid wash of the ethyl acetate extract to the aqueous amine extract (p.197) had the effect of increasing the noradrenaline estimates in each area of brain by about one third (p.216). This proportionate increase is of the same order as the proportion of noradrenaline found in the acid washes of the ethyl acetate extracts of three hypothalamic acid extracts (p.199) and is too great to be due solely to mechanical carry-over of the amine from the aqueous, into the ethyl acetate and then back into the aqueous, phase. The amounts of dopamine, equivalent to about 3% of the dopamine present in the aqueous phase, which were found in the acid washes from ethyl acetate extracts of three acid extracts of caudate nucleus, were low enough to be explained solely on the basis of a mechanical carry-over. Under the extraction conditions employed (p. ), noradrenaline, but not dopamine, would appear to be transported into ethyl acetate, presumably attached to some ethyl acetate-soluble substance, since noradrenaline is itself not extracted into ethyl acetate (p.121).

Extraction of noradrenaline from acid solution into ethereal solutions of 'tissue lipids', lecithin and

cephalin was reported by von Euler (1946, 1947). The noradrenaline could then be back-extracted from the ether lipid into 5% sodium sulphate solution (von Euler, 1946, 1947). A similar mechanism may be responsible for the removal into ethyl acetate of some of the noradrenaline in an acid (pH about 2) extract of dog brain and for the subsequent back-extraction of this noradrenaline from the ethyl acetate into a salt-saturated solution of 0.01N hydrochloric acid (p. 190). Lecithin and cephalin are both present in dog brain (Biochemist's Handbook, 1961, p. 644). No reports, however, could be found in the literature regarding the ability of ethyl acetate solutions of these or any other tissue lipids, to remove noradrenaline from acid aqueous extracts (p. // ). It is difficult to reconcile the relatively high degree of extraction of noradrenaline (equivalent to about 30% of the total noradrenaline in the aqueous phase) from brain extracts at pH 2 into ethyl acetate, with the estimate of 5% quoted by Norlander (1950) for the extraction of noradrenaline into ethereal solutions containing 5% lecithin from an aqueous solution at pH 4. Reconciliation is especially difficult since von Euler (1947) and Norlander (1950) found the percentage of noradrenaline extracted was reduced if the pH of the aqueous phase was less than 4. These workers, however, only investigated the degree of



extraction of noradrenaline into lecithin-ether, from solutions containing milligram amounts of noradrenaline; it is possible that proportionately more noradrenaline is extracted from solutions containing microgram or milli-microgram amounts of noradrenaline. It is also possible that the postulated noradrenaline-lipid complex formed from brain tissue is stable at a lower pH than the noradrenaline-lecithin complex and is more readily extracted into ethyl acetate than is the noradrenaline-lecithin complex into ether.

Unfortunately, a second extraction with salt saturated 0.01N hydrochloric acid of the ethyl acetate fraction was never carried out. It is therefore not possible to be certain that all the noradrenaline removed into the ethyl acetate was recovered into a single back-wash. The noradrenaline estimates obtained by incorporating only a single back-wash into the amine fraction may therefore be under-estimates.

A different person was responsible for the brain dissections in the experiments of group 1 from that in the experiments of group 2. It is possible that this difference could have contributed to the increased levels of noradrenaline found in the experiments of group 2, in which the acid wash from the ethyl acetate fraction was combined with the aqueous, amine fraction. There is, unfortunately, no way of testing for the influence



of dissectors on the amine estimates within the experimental design used. It is, however, highly unlikely that each area of brain dissected by one operator would be so exactly different from the same area dissected by the other operator as to cause a consistent 30% - 50% increase in the noradrenaline concentration of each brain area.

#### Recovery of the Catechol- and Methoxy-amines through the Procedure

No satisfactory explanation could be found for the observation that catechol- and methoxy-amines added to 0.4N perchloric acid extracts of brain tissue were, unlike the corresponding endogenous amines, subject to extremely variable degrees of loss or destruction during the extraction procedure. The results of the control experiments (p. 218) indicated that the losses might occur at two stages in the extraction procedure as a result of (i) adsorption onto the protein precipitate which was subsequently discarded and (ii) destruction in the acid supernatant or during the subsequent adjustment of its pH from 0.8 to 4 (Table 21, p. 217). Since 200 mug amounts of adrenaline and noradrenaline were quantitatively recovered from pure solutions of 0.1N perchloric acid (p. 122), it is unlikely that loss of amines added to the 0.4N perchloric acid brain extract

was due to the perchloric acid itself, as suggested by the work of Gunne (1963, p. 10 ), or to the subsequent pH adjustment.

Anton and Sayre obtained 80% recovery of 0.2 ug to 1.0 ug noradrenaline added to 0.4N perchloric acid homogenates of rat liver (1962) and 80% recovery of 0.2 ug to 2.0 ug dopamine added to 0.4N perchloric acid homogenates of dog brain (1964). Gunne (1963) recovered 98%, 76% and 80% of 1.0 ug amounts of noradrenaline, adrenaline and dopamine, respectively, added to perchloric acid extracts of brain. Both Anton and Sayre and Gunne stated that the temperature during homogenisation was kept at 0°-4°C but they did not specify if this temperature was maintained during addition of the amines and adjustment of the pH. Without this knowledge, their results cannot be compared with those given on p.220, in which it was discovered that loss of amines added to the 0.4N perchloric acid extract was prevented if the extract was maintained at about 0°C during addition of the amines and during the subsequent pH adjustment. Since the replicate estimates for rat brain amines (p.174) and for dog brain amines (p. 20) were reasonably consistent despite varying recoveries of exogenous amines, it would appear that the endogenous amines must be protected in some way from those influences causing the losses of the exogenous

amines. This protection might be afforded by an attachment to a protein or lipid (Barger, 1930; von Euler, 1947), the combination being stable in, and not precipitated by, the perchloric acid. It is difficult to envisage the nature of such a complex since perchloric acid is an efficient protein precipitant (Neuberg, Strauss and Lipkin, 1944) and the resultant extract has a relatively low pH, a condition under which any complex is unlikely to be stable. There is a possibility, albeit remote, that the endogenous amines under these extraction conditions are present in some occluded state. It would be of interest in this connection to know the efficacy of perchloric acid in releasing catecholamines from storage organelles in vitro.

Distribution of Catechol and Methoxy-amines  
in Dog Brain

In Table 22, are listed the estimates, made by various other workers and by the author, of the catechol- and methoxy-amine concentrations in discrete areas of dog brain. The quoted values for noradrenaline in the present series are those from the second group of experiments since they include some noradrenaline known to have been lost in the experiments in group 1 (p.222). Our values for noradrenaline in the midbrain, cortex and thalamus agree fairly closely with the estimates

quoted in the literature (Table 22). Our estimate for noradrenaline in hindbrain is similar to that given by Bertler and Rosengren (1959) for noradrenaline in the medulla oblongata, which formed the major part of our hindbrain section (Table 15, p. 186). Our estimate for noradrenaline in the hypothalamus is, however, lower than the estimates quoted by other workers. It may be that 1 - 2 year old beagles have less noradrenaline in their hypothalami than the dogs (breed unspecified), used by other workers. Although, as reported by Vogt (1954), the noradrenaline estimates for each area were found to vary widely from dog to dog, the pattern of distribution within one dog remained the same.

Adrenaline was found at a concentration less than that reported by Vogt (1954) and Malhotra and Prasad (1962) in the hypothalamus, but in no other area of dog brain. The fact that adrenaline equivalent to about 14% of the noradrenaline concentration was present in all high noradrenaline-containing areas (Vogt, 1954) suggests that adrenaline can be formed from noradrenaline in brain. McGeer and McGeer (1964) isolated  $H^3$ -adrenaline and  $C^{14}$ -adrenaline from cat brain stem after injection of  $H^3$ -noradrenaline and  $C^{14}$ -tyrosine, respectively, into the brain stems of anaesthetised cats, showing that brain tissue has the capacity to synthesise



TABLE 22

Mean Estimates of Adrenaline, Noradrenaline and Dopamine ( $\mu\text{g/g}$ ) in discrete Areas of Dog Brain as obtained by different Workers.

	Caudate Nucleus		Hypothalamus			Midbrain		Cortex		Thalamus		Medulla Oblongata	
	NA	DA	A	NA	DA	NA	DA	NA	DA	NA	DA	NA	DA
Bertler and Rosengren (1959) (fluorimetric assay)	0.10	5.90	N.D.	0.76	0.26	N.D.	N.D.	0.12	0.07	N.D.	N.D.	0.37	0.13
Lavery and Sherman (1965) (fluorimetric assay)	0.09	9.9	N.D.	1.35	0.25	0.43	0.33	0.16	0.01	N.D.	0.05	N.D.	N.D.
Vogt (1954) (biological assay)	0.06	N.D.	0.17	0.6-1.76 (range)	N.D.	0.24-0.60 (range)	N.D.	0.04 (visual) 0.08 (frontal)	N.D.	0.23 (medial) 0.08 (lateral)	N.D.	N.D.	N.D.
Malhotra and Prasad (1962) (biological assay)	N.D.	N.D.	0.15	0.92	N.D.	0.28	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Present Author	< 0.18	5.5	0.05	0.59	0.21	0.44	0.14	0.08 (occipital)	< 0.04 (occipital)	0.20	0.04	0.28 (hind-brain)	0.02 (hind-brain)

A = adrenaline; NA = noradrenaline; DA = dopamine

N.D. = not estimated.

adrenaline. Phenylethanolamine-N-methyl-transferase, an enzyme capable of methylating noradrenaline to produce adrenaline has been detected in 'minute amounts' in rabbit midbrain (Axelrod, 1962) and in cat hypothalamus and caudate nucleus (McGeer and McGeer, 1964).

No estimates for metanephrine in brain could be found in the literature although the detection of radioactively labelled metanephrine in rat brain after intra-ventricular administration of radioactively labelled adrenaline (Snyder, Glowinski and Axelrod, 1965) suggests that brain is capable of synthesising metanephrine from adrenaline.

As reported by Bertler and Rosengren (1959), the distribution of dopamine in dog brain was found to be quite different from that of noradrenaline. Our estimate for dopamine in the caudate nucleus (5.5 ug/g) agrees closely with Bertler and Rosengren's estimate of 5.9 ug/g but both are lower than that found by Lavery and Sharman (1965). In both the caudate nucleus and the globus pallidus the concentration of dopamine was five to twenty times the concentration of noradrenaline. This high concentration of dopamine <sup>in</sup> relative to noradrenaline/the basal ganglia of the dog has been found in other species (p.178), and is suggestive of a transmitter function for dopamine in the basal ganglia.

The estimate obtained for methoxydopamine in dog caudate nucleus (0.61  $\mu\text{g/g}$ ) is of the same order as estimates found by Carlsson and Waldeck (1964) for methoxydopamine in the caudate nuclei of pigs and sheep (0.83  $\mu\text{g/g}$  and 1.2  $\mu\text{g/g}$ , respectively). About the same proportion of methoxydopamine to dopamine was found in the globus pallidus as in the caudate nucleus, implying a similar rate of metabolism of dopamine by catechol-o-methyl-transferase in these two basal ganglial areas of dog brain.

Effect of Chlorpromazine on the Metabolism of the Catechol-  
amines in Dog Brain

Effect of Chlorpromazine on the Metabolism of Dopamine  
in the Caudate Nucleus

The effect of chlorpromazine on dopamine metabolism will be discussed using the estimates obtained from the caudate nucleus, because chlorpromazine produced statistically significant changes in the concentrations of dopamine and its metabolites in this area. Although chlorpromazine also altered the concentrations of dopamine and its metabolites in other areas, these alterations were, with certain exceptions, not statistically significant ( $p > 0.10$ ).



Effect of chlorpromazine on the distribution of dihydroxyphenylacetic acid and homovanillic acid between the caudate nucleus and lateral ventricular

C.S.F... The increase in the concentrations of dihydroxyphenylacetic acid and homovanillic acid in the caudate nucleus produced by 5 mg/kg chlorpromazine was proportionately the same as the increase in the concentrations of these acid metabolites in the lateral ventricular C.S.F. produced by this same dose of chlorpromazine (Guldborg, personal communication). This is evidence in favour of the hypothesis (p. 182) that the levels of dihydroxyphenylacetic acid and homovanillic acid found in the lateral ventricular C.S.F. reflect those in the caudate nucleus. The ratios of the brain acid levels to the C.S.F. acid levels were unaltered by treatment of the dog with 5 mg/kg or 15 mg/kg chlorpromazine, indicating that such doses of the drug did not affect the transfer of the acid metabolites from the caudate nucleus into the C.S.F..

Effect of 5 mg/kg chlorpromazine on the metabolism of dopamine in the caudate nucleus. Administration of 5 mg/kg chlorpromazine increased the concentrations of the acid metabolites of dopamine, decreased the methoxydopamine and had no effect on the dopamine concentration. With the exception of the alteration in methoxydopamine, all these actions of chlorpromazine

had been demonstrated previously in the corpus stratum of the rabbit (Andén, Roos and Werdinius, 1964). The rise in the levels of the acid metabolites cannot be explained by a release of dopamine from its storage sites onto monoamine oxidase, which is believed to account for the metabolism of amines released intraneuronally (Glowinski and Axelrod, 1965), because there was no concomitant reduction in the dopamine concentration. Two possible explanations for these effects of chlorpromazine remain namely:

- (i) stimulation of dopamine turnover;
- (ii) inhibition of acid egress from the caudate nucleus.

(i) Stimulation of dopamine turnover. An increase in dopamine synthesis could cause an increase in the levels of dihydroxyphenylacetic acid and homovanillic acid.

A rise in the dopamine level need not necessarily result from such an increased synthesis since the dopamine stores may be already saturated. Since there is no way of distinguishing 'stored' from 'free' dopamine, it is impossible to tell if the dopamine stores under normal conditions are saturated or not. Other possible explanations for the unchanged dopamine levels are that there is an inhibition of uptake into and release of dopamine from, storage sites or that there is an activation of mitochondrial monoamine oxidase, so that newly synthesised dopamine is metabolised by monoamine

oxidase instead of being stored. There is in vitro evidence that chlorpromazine, at a concentration that might be expected in brain following a 5 mg/kg dose (de Jaramillo and Guth, 1963) inhibits uptake of dopamine by rabbit and bovine adrenal medullary granules (Carlsson, Hillarp and Waldeck, 1962) and inhibits release of adrenaline from rabbit adrenal medullary granules (Weil-Malherbe and Posner, 1963). It is not, however, known if dopamine exists in the brain in membrane limited organelles (p. 179). The possible activation of mitochondrial monoamine oxidase by low concentrations of chlorpromazine was suggested by Guldberg (1967, p. 112), but has not been examined experimentally.

(ii) Inhibition of acid egress from the caudate nucleus. We found that 5 mg/kg chlorpromazine did not affect the transfer of homovanillic acid or dihydroxyphenylacetic acid from the brain tissue into the C.S.F. (p.231). It is, however, possible that chlorpromazine blocks transport of these acids from the brain and from the C.S.F. into the blood. Andén, Roos and Werdinius (1964) and Sharman (1967) have also obtained evidence that chlorpromazine may have such an inhibitory action. A method of investigating such a mode of action would be to estimate homovanillic acid in the lateral ventricular C.S.F. and cisternal C.S.F., before and after

administration of chlorpromazine. Since homovanillic acid has been shown to be actively removed from the C.S.F. at some point between the lateral ventricle and the cisterna (Guldborg, Ashcroft and Crawford, 1966), any effect of chlorpromazine on active transport would be reflected in an alteration of the ratio of the concentrations of the acids in the lateral ventricular and cisternal C.S.F.

Although, in view of the increase in the noradrenaline levels in other areas, a stimulation of catecholamine synthesis would appear to be the more likely explanation for the action of 5 mg/kg chlorpromazine leading to an increase in the acid metabolites, it is not possible, on the present evidence, to exclude an inhibitory action on the egress of the acid metabolites from brain.

The marked fall in the levels of methoxydopamine after 5 mg/kg chlorpromazine is unlikely to be due to inhibition of catechol-O-methyl-transferase since this inhibition would also result in a decrease in the levels of homovanillic acid. A reduction in methoxydopamine could be brought about by an inhibition of extraneuronal release of dopamine or by the facilitated conversion of methoxydopamine to homovanillic acid. As already stated (p.233), low doses of chlorpromazine may inhibit uptake of dopamine into and release of dopamine from,

its storage sites and such doses may activate mitochondrial monoamine oxidase.

The mechanism of a chlorpromazine induced increase in catecholamine synthesis is a matter for speculation. Activation of tyrosine hydroxylase has been demonstrated in the brains of rats following intraperitoneal administration of 10 mg/kg chlorpromazine (Burkard, Gey and Pletscher, 1967) but it is not known if this is a direct or an indirect action. Carlsson and Lindquist (1963) and Andén, Roos and Werdinius (1964) suggested that chlorpromazine acts as an adrenergic blocking agent in the brain, as in the periphery (Courvoisier, Fournel, Ducrot, Kolsky and Koetschet, 1953) and that block of central adrenergic receptors resulted in an increased release of catecholamines from the nerve endings, which in turn stimulated catecholamine synthesis. Carlsson and Lindquist (1963), using mice pre-treated with a monoamine oxidase inhibitor, found that 5 mg/kg chlorpromazine gave a rise in the concentrations of normetanephrine and methoxydopamine in brain, without affecting the levels of noradrenaline and dopamine. These findings are in accord with the hypothesis that chlorpromazine increases dopamine turnover but do not necessarily imply, as Carlsson and Lindquist inferred, that chlorpromazine stimulates this turnover by blocking adrenergic receptors. Andén et al. (1964) deduced,

since Carlsson and Lindquist (1963) had found that the levels of normetanephrine but not those of methoxydopamine, were increased by another  $\alpha$ -adrenergic blocking agent namely phenoxybenzamine, that chlorpromazine stimulated dopamine synthesis in the corpus striatum as a consequence of blocking receptors which "were not of the  $\alpha$  type". Our results with a higher dose of chlorpromazine (15 mg/kg) make it unlikely that chlorpromazine stimulates synthesis as a result of receptor block. Presumably 15 mg/kg chlorpromazine could be expected to give a more intense block of the catecholamine receptors and effect an even greater release of dopamine and stimulation of dopamine synthesis, than would be effected by 5 mg/kg. The fact that 15 mg/kg had no effect on the concentrations of the acid metabolites suggests that this dose of chlorpromazine does not increase catecholamine synthesis. Stimulation of synthesis by 15 mg/kg chlorpromazine to the point of exhaustion of the substrate is unlikely, in view of the relatively high concentration in the brain of the catecholamine precursor, tyrosine (Tallan, Moore and Stein, 1954).

Effect of 15 mg/kg chlorpromazine on the metabolism of dopamine in the caudate nucleus. 15 mg/kg chlorpromazine brought about a reduction in the levels of dopamine and methoxydopamine but did not alter those



of the acid metabolites of dopamine. Lavery and Sharman (1965) also found a reduction in the dopamine content of cat caudate nucleus, 4 hr. after the subcutaneous administration of 10 mg/kg chlorpromazine. Release of catecholamines from bovine (Carlsson and Hillarp, 1961) and rabbit (Weil-Malherbe and Posner, 1963) adrenal medullary granules in vitro has been demonstrated with concentrations of chlorpromazine greater than those required to inhibit catecholamine uptake and release (p.233). These facts suggest that 'high' doses of chlorpromazine may release brain dopamine from its storage sites. As a consequence of the reduction in stored dopamine, less is available for release upon nervous stimulation and the concentration of methoxydopamine therefore falls. The fact that reserpine, which decreases the levels of noradrenaline and dopamine in brain (Bertler, 1961) also lowers the concentrations of normetanephrine and methoxydopamine in brain (Häggendal, 1963a), suggests that a reduction in the methoxyamines may be a consequence of a reduction in stored catecholamines.

In order to explain the unchanged levels of dihydroxyphenylacetic acid and homovanillic acid occurring simultaneously with release of dopamine, it is necessary to make one or other of the following postulates regarding the action of 15 mg/kg chlorpromazine:

- (i) facilitation of the egress of the dopamine acid metabolites from brain;
- (ii) metabolism, by a different pathway, of the dopamine released by chlorpromazine;
- (iii) decrease in dopamine synthesis.

It should be possible to investigate (i) in the same way as was suggested to investigate the hypothesis that 5 mg/kg chlorpromazine inhibited acid egress (p.233 ). (ii) Inhibition of aldehyde dehydrogenase by chlorpromazine with the consequent formation of the alcohols, instead of the acids, from the 3:4: dihydroxyphenylacetaldehyde formed from dopamine by the action of monoamine oxidase, may take place. (iii) A decrease in dopamine synthesis would follow if the drug diminished uptake of the precursor amino acid, tyrosine. Lajtha and Toth (1965) found that 50 mg/kg chlorpromazine inhibited uptake of amino acids by mouse brain in vivo.

#### Effect of Chlorpromazine on the Catecholamines and their Metabolites in Areas other than the Caudate Nucleus

Chlorpromazine (5 mg/kg) had no significant effect on the levels of dopamine and its metabolites in the globus pallidus. This lack of significance could be due to the wide scatter in the estimates, which may be a reflection of the difficulty in dissecting this area reproducibly. However, the fact that 15 mg/kg chlorpromazine significantly lowered the dopamine concentration

( $p < 0.10$ ) suggests that, at least at the high dose, chlorpromazine affected dopamine metabolism in the globus pallidus in the same way as it affected dopamine metabolism in another basal ganglia, the caudate nucleus.

The noradrenaline concentrations in those areas found to contain more noradrenaline than dopamine, namely the hypothalamus, thalamus, midbrain and hindbrain, were all increased by the low and also by the high dose except in the case of the midbrain. However, only in the thalamus were the increases significant ( $p < 0.01$ ). Since no estimates were obtained for the metabolites of noradrenaline, it was impossible to say if these increased levels of noradrenaline were due to a decrease in noradrenaline release or to an increase in noradrenaline synthesis. However, the fact that the dopamine concentrations in the hypothalamus and thalamus were increased by 5 mg/kg chlorpromazine suggests, as has been postulated to occur in the caudate nucleus, that this dose of chlorpromazine stimulates catecholamine synthesis. Whereas in the caudate nucleus this stimulation resulted in increases in the metabolism of dopamine to its acid metabolites, in the noradrenaline-containing areas the increased synthesis of dopamine was channelled into production of more noradrenaline in the noradrenaline storage granules (Kirschner, 1959) and the levels of dihydroxyphenylacetic

acid and homovanillic acid were not significantly altered. The fact that the noradrenaline concentrations were increased by 15 mg/kg chlorpromazine indicates that this dose may also increase noradrenaline synthesis.

There was no specific effect of chlorpromazine (15 mg/kg) on the levels of the amines and their metabolites in the hindbrain which could be correlated with the observation of Bradley and Key (1959) that chlorpromazine increased the threshold for arousal produced by stimulation of the brain stem reticular formation.

The thalamus is of particular interest since, alone among the areas which contained more noradrenaline than dopamine, the increases in noradrenaline concentration produced by both doses of chlorpromazine were highly significant ( $p < 0.01$ ). The concentration of dopamine in the thalamus was also significantly increased ( $p < 0.02$ ) by 5 mg/kg chlorpromazine. This could imply that the thalamus is very susceptible to the stimulation of catecholamine synthesis induced by chlorpromazine. No information on the rate of turnover of catecholamines in the thalamus could be found in the literature.

An increase in the noradrenaline concentration was observed in the hypothalamus and midbrain after treatment with both doses of chlorpromazine. Malhotra and Prasad (1962) have also reported increases in the noradrenaline concentrations in these areas after

5 mg/kg chlorpromazine. The increases observed by them were, however, much greater than those found by us. They did not investigate the effect of 15 mg/kg but reported that 10 mg/kg produced a fall in the noradrenaline concentrations. The following are possible reasons for the discrepancies between their results and our results: (i) they used chloroform as an anaesthetic whereas we used thiopentone; (ii) they did not control the body temperature of the dogs during treatment with chlorpromazine; (iii) they probably did not use the same breed of dogs as was used by us.

To conclude, it would appear that the fundamental action of chlorpromazine in brain is to stimulate catecholamine synthesis. Other, apparently dose dependent, actions operate to alter the metabolic pattern of the amines and their metabolites in the brain, following administration of different doses of the drug.

## SUMMARY

Methodology

The aim was to develop a method whereby the catecholamines, their precursors and metabolites could be estimated in the same brain sample. Since the acid metabolites could be removed from a brain extract by extraction into an organic solvent and subsequently estimated, the main problem centred on devising a technique which would allow the estimation of the catecholamines, methoxyamines and dopa present in the brain extract after removal of the acid metabolites. Two techniques were devised and have been discussed. The first depended on the separation of the amines and dopa by paper chromatography in phenol:hydrochloric acid prior to estimation. This method proved unsatisfactory and was superseded by a more satisfactory method, in which the amines and dopa were separated and estimated as their acetyl derivatives.

The phenol:hydrochloric acid method. Rat brain was homogenised in perchloric acid and perchloric acid removed as its insoluble potassium salt. The extract was chromatographed on paper, first in an acidified saturated sodium chloride solution to separate the catecholamine group from the indolic substances.



After removal of that portion of the chromatogram bearing the indoles, chromatography was continued in phenol:hydrochloric acid. The three methoxy amines were separated from one another and from the catechol compounds. Noradrenaline was not well separated from dopa; these substances were therefore eluted together and estimated by a differential fluorimetric method. For a similar reason, adrenaline and dopamine were eluted together and estimated by a differential method.

Consistent estimates were obtained for noradrenaline in rat brain but the estimates for rat brain dopamine and normetanephrine were unexpectedly high.

The method proved unsatisfactory in practice for the following reasons:

- (i) The presence of phenol in the chromatogram eluates complicated the assays.
- (ii) It was suspected that some other substance was included in the dopamine estimates.
- (iii) The assay method for dopamine was insufficiently sensitive.
- (iv) There was on occasion incomplete chromatographic separation of normetanephrine and adrenaline, necessitating estimations in two separate portions of eluate, thus decreasing the sensitivity of the determination.

Separation and estimation of the amines as their acetylated derivatives. The technique outlined below was rigorously tested in pure solution before its application to the estimation of the amines and their metabolites in extracts of rat brain was investigated.

The brain tissue was homogenised in perchloric acid and the perchloric acid removed as the potassium salt from the supernatant.

The acid metabolites of the amines were extracted from the acidified extract into ethyl acetate, back-extracted into 'tris' buffer and aliquots taken for the fluorimetric estimation of homovanillic acid and dihydroxyphenylacetic acid.

After extraction with ethyl acetate, the acid aqueous extract was acetylated and the acetylated derivatives of the amines extracted into dichloromethane.

The aqueous remaining after extraction with dichloromethane was acidified and the acetylated derivatives of the amino acids extracted into ethyl acetate. The acetyl-dopa in this extract was estimated by the ethylenediamine fluorimetric method.

The dichloromethane extract containing the acetylated amines was chromatographed on paper in the organic phase of toluene : ethyl acetate : methanol : water = 10 : 1 : 5 : 5.

The acetylated amines were eluted and assayed fluorimetrically.

Acetyl-noradrenaline, acetyl-adrenaline and acetyl-dopamine were estimated after condensation with ethylenediamine. Acetyl-metanephrine and acetyl-normetanephrine were estimated after acid hydrolysis, by the tri-hydroxyindole procedure.

A new method, based on coupling with ethylenediamine, was developed for the estimation of acetyl-methoxy-dopamine (and methoxydopamine).

The sensitivities of assay for the different acetylated amines in pure solution ranged from 10  $\mu$ g to 45  $\mu$ g.

This technique gave consistent estimates for noradrenaline, dopamine and methoxydopamine in rat brain. Adrenaline and normetanephrine were below the level of detection of the method. The recoveries of the amines added to perchloric acid extracts of brain tissue were not satisfactory. The reasons for this have been investigated.

The analytical method was applied to a study of the effects of chlorpromazine (CPZ) at two dose levels, 5 mg/kg and 15 mg/kg, intravenously, on the catecholamine metabolism in various areas of the brains of beagle dogs. Two hours after drug administration, the following changes were observed.

Dopamine: 5 mg/kg CPZ produced no change in the levels in the caudate nucleus, globus pallidus and midbrain and increased the levels in the hypothalamus ( $p < 0.10$ ) and in the thalamus ( $p < 0.02$ ).

15 mg/kg CPZ gave a reduction in the levels in the caudate nucleus and globus pallidus ( $p < 0.10$ ). The concentrations in the hypothalamus, midbrain, thalamus and hind-brain were unchanged.

Homovan- 5 mg/kg CPZ increased the levels of both  
illic acid acids in the caudate nucleus ( $p < 0.05$ ) and  
and had no effect on the acid concentrations  
Dihydroxy- in the globus pallidus, hypothalamus and  
phenyl- thalamus.

acetic acid: 15 mg/kg CPZ produced no change in the  
acid concentrations in any area.

Methoxy- 5 mg/kg and 15 mg/kg CPZ reduced the  
dopamine: concentration in the caudate nucleus  
( $p < 0.05$ ) and had no significant effect  
on the concentration in the globus pallidus.

Nor- The concentrations in the hypothalamus,  
adrenaline: midbrain, thalamus and hindbrain were  
increased by 5 mg/kg and 15 mg/kg CPZ.  
However, only in the case of the thalamus  
( $p < 0.01$ ) were these increases significant.

It was concluded that the actions of chlorpromazine on catecholamine synthesis and metabolism in the brain of the dog are dose dependent.

In the lower dose of 5 mg/kg, chlorpromazine was postulated to have the following actions:-

- (1) to increase catecholamine synthesis;
- (2) to inhibit the egress of the acid metabolites of dopamine from the brain tissue;
- (3) to activate mitochondrial monoamine oxidase or to inhibit extraneuronal release of dopamine.

At the higher dose level of 15 mg/kg, chlorpromazine was postulated to act as follows:

- (1) to release dopamine from its storage sites;
- (2) to alter the metabolic pathway of dopamine or to potentiate egress of the acid metabolites of dopamine from the brain tissue;
- (3) to increase catecholamine synthesis in those areas of brain containing higher concentrations of noradrenaline than of dopamine.

The fundamental action of chlorpromazine in brain was concluded to be a stimulation of catecholamine synthesis.

## APPENDIX

Estimation of Acetyl-dopa

The low sensitivity of the estimation of acetyl-dopa in the presence of brain extracts (p. 160) was due to the following factors:

- (i) a high 'blank' fluorescence, originating from the residue from evaporation of the 20 - 30 ml ethyl acetate used to extract the acetylated acid (p. 156),
- (ii) the quenching of acetyl-dopa fluorescence by the tissue extracts (p. 160),
- (iii) the low intensity of the fluorescence derived from acetyl-dopa, as was indicated by the fact that the sensitivity of estimation of acetyl-dopa in pure solution was only 40 mug (Table 8, p. 126).

Using the following technique, quenching by tissue extracts was eliminated and the fluorescence from a tissue extract to which no dopa had been added was reduced from 5 times to 3 times, that from the reagent blank. This resulted in a sensitivity of estimation of acetyl-dopa in the presence of a tissue extract, of 120 mug, as compared with 400 mug using the method described in Section I (p. 119).

The acetyl-dopa was extracted into ethyl acetate from the acidified aqueous solution after removal of the acetylated amines (p. 118). To the ethyl acetate

(20 - 30 ml) was added 2.5 ml 0.1M pH 8.3 borate buffer together with solid sodium bicarbonate (0.75 g per 0.5 ml acetic anhydride added during the acetylation procedure). This amount of sodium bicarbonate was required to neutralise the acetic acid contained in the ethyl acetate (p. 119). Vigorous stirring for 15 - 20 min. was essential to ensure that reaction of the acetic acid with the sodium bicarbonate went to completion. Borate buffer was used because there was an indication that it slightly potentiated the fluorescence from acetyl-dopa. To 1.0 ml of this borate extract was added 100  $\mu$ g acetyl-dopa as an internal standard, another 1.0 ml portion being used as the sample. The fluorescence was developed as on p. 119. A mean recovery of  $78\% \pm 16\%$  (S.D.) was obtained from 0.5  $\mu$ g dopa added to extracts from six different areas of dog brain just before the acetylation procedure. The high 'blank' fluorescence (p. ) was probably due to the high concentration in the extract solution, of sodium acetate resulting from the neutralisation of the acetic acid (about 1 ml 10M) present in the ethyl acetate. Acetyl-tyrosine, which might be present in the acetyl-dopa extract, would not contribute to the 'blank' fluorescence because it did not react with ethylenediamine.



On further investigation, dichloromethane was found to extract acetyl-dopa from acid solution with the same efficiency as ethyl acetate. However, as was found with the ethyl acetate extracts derived from brain tissue, evaporation of the dichloromethane extracts to dryness and estimation of acetyl-dopa in the residues gave high blanks and about 50% quenching of the fluorescence from added acetyl-dopa. Although dichloromethane took up very much less of the acetic acid, formed by interaction of hydrochloric acid with the sodium acetate (p.119) formed during the acetylation procedure, than did ethyl acetate, sufficient was removed into a 20 ml dichloromethane extract to prevent back-extraction of acetyl-dopa into 2.5 ml 0.1M pH 8.3 borate buffer. However, less sodium bicarbonate would be required to neutralise the acid contained in the dichloromethane extract with a resulting decrease in the amount of sodium acetate, compared with that from ethyl acetate extracts, in the aqueous phase for analysis and hence a possible lowering of the 'blank' fluorescence. This modification remains to be investigated.

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